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Recognition of seven species in the *Cryptococcus gattii*/*Cryptococcus neoformans* species complex

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ABSTRACT

Phylogenetic analysis of 11 genetic loci and results from many genotyping studies revealed significant genetic diversity with the pathogenic *Cryptococcus gattii*/*Cryptococcus neoformans* species complex. Genealogical concordance, coalescence-based, and species tree approaches supported the presence of distinct and concordant lineages within the complex. Consequently, we propose to recognize the current *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* as separate species, and five species within *C. gattii*. The type strain of *C. neoformans* CBS132 represents a serotype AD hybrid and is replaced. The newly delimited species differ in aspects of pathogenicity, prevalence for patient groups, as well as biochemical and physiological aspects, such as susceptibility to antifungals. MALDI-TOF mass spectrometry readily distinguishes the newly recognized species.

1. Introduction

Cryptococcus neoformans (Sanfelice) Vuillemin is a clinically important basidiomycetous yeast that globally causes an estimated 1 million new infections and over 625,000 deaths annually. Although this estimate has recently been disputed, the species remain a main source of morbidity and mortality (Park et al., 2009, 2014a; Warkentien and Crum-Cianflone, 2010). According to the current classification the species complex comprises two species, namely *C. neoformans* and *C. gattii* (Vanbreuseghem & Takashio) Kwon-Chung & Boekhout (Kwon-Chung et al., 2002; Kwon-Chung and Varma, 2006) with serotypes A, D and AD for the former, and B and C for the latter species. *Cryptococcus neoformans* currently consists of two varieties: *C. neoformans* variety *grubii* (serotype A) (Franzot et al., 1999) and *C. neoformans* variety *neoformans* (serotype D) (Kwon-Chung, 2011). With the dual nomenclature that was in use in fungal taxonomy until recently, the teleomorphs (i.e., the sexual stages) were named *Filobasidiella neoformans* Kwon-Chung and *Filobasidiella bacillispora* Kwon-Chung (Franzot et al., 1999; Kwon-Chung, 1975, 1976, 2011; Kwon-Chung et al., 2002; Vanbreuseghem and Takashio, 1970). A detailed account on the history of our knowledge of the species was presented by Drouhet (1997), Barnett (2010) and Kwon-Chung et al. (2011) as well as in two books (Casadevall and Perfect, 1998; Heitman et al., 2011). A recent review on *C. gattii* infections highlighted the history, epidemiology and clinical impact of that species and its subtypes (or species) (Chen et al., 2014).

During the past two decades, considerable genetic heterogeneity has been demonstrated to occur in the *C. gattii*/*C. neoformans* species complex by a plethora of molecular methods, such as multi-locus enzyme typing (Brandt et al., 1993), amplified fragment length polymorphism (AFLP) (Boekhout et al., 2001; Hagen

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et al., 2010a, 2012b; Kidd et al., 2004; Meyer et al., 2011), PCR-fingerprinting using M13, $(GACA)_4$ and $(GTG)_5$ primers (Meyer et al., 1993, 1999; Meyer and Mitchell, 1995; Sorrell et al., 1996; Viviani et al., 1997), random amplification of polymorphic DNA (RAPD) (Aoki et al., 1999; Boekhout and Van Belkum, 1997; Meyer et al., 1999; Sorrell et al., 1996), restriction fragment length polymorphism fingerprinting based on the genes *CAP10* (Raimondi et al., 2007), *CAP59* (Enache-Angoulvant et al., 2007; Raimondi et al., 2007), *GEF1* (Feng et al., 2008a), *PLB1* (Latouche et al., 2003) and *URA5* (Casadevall et al., 1992; Kidd et al., 2004; Meyer et al., 2003), Fourier transform infrared-spectroscopy-based (FTIR) phenotyping (Lemmer et al., 2004), multi-locus microsatellite typing (Hagen et al., 2012a, 2013; Hanafy et al., 2008; Illnait-Zaragozí et al., 2010; Karaoglu et al., 2008; Pan et al., 2012), sequence analysis of a considerable number of genes (Bovers et al., 2008a, 2009; Butler and Poulter, 2005; Chen et al., 2014; Diaz et al., 2000, 2005; Fraser et al., 2005; Hagen et al., 2012b, 2013; Katsu et al., 2004; Khayhan et al., 2013; Litvintseva et al., 2006; Meyer et al., 2009; Ngamskulrungroj et al., 2009; Simwami et al., 2011; Springer et al., 2014; Sugita et al., 2001; Xu et al., 2000, 2009), as well as sequence divergences based on whole genome analyses (Billmyre et al., 2014; D'Souza et al., 2011; Engelthaler et al., 2014; Loftus et al., 2005; Voelz et al., 2013). The results of these studies strongly question the currently used two species concept in the *C. gattii*/*C. neoformans* species complex.

In addition, considerable differences have been documented between the *C. neoformans* complex on the one hand and the *C. gattii* complex on the other. For instance, the varieties *grubii* and *neoformans* of *C. neoformans* differ from *C. gattii* in their electrophoretic karyotypes (Boekhout et al., 1997; Wickes et al., 1994), DNA-fingerprints (Varma et al., 1995), intergenic spacer sequences (IGS) of the ribosomal DNA (Diaz et al., 2000, 2005), physiological and biochemical characteristics (Bennett et al., 1978; Cherniak and Sundstrom, 1994; Dufait et al., 1987; Kwon-Chung et al., 1987; Mukamurangwa et al., 1995; Ngamskulrungroj et al., 2012a; Polacheck and Kwon-Chung, 1980), mass spectra generated by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) profiles (Firacative et al., 2012; Hagen et al., 2011; McTaggart et al., 2011; Posteraro et al., 2012), susceptibility to killer toxins of *C. laurentii* CBS139 (Boekhout and Scorzetti, 1997), geographic distribution and habitat (Bennett et al., 1977; Cogliati, 2013; Hagen et al., 2012b; Kwon-Chung and Bennett, 1984a,b; Meyer et al., 2011; Springer and Chaturvedi, 2010), antifungal susceptibility profiles (Chowdhary et al., 2011; Espinel-Ingroff et al., 2012a,b, 2015; Hagen et al., 2010a, 2012a; Iqbal et al., 2010; Lockhart et al., 2012; Pan et al., 2012), induction of cytokines (Schoffelen et al., 2013), pathophysiology (Capilla et al., 2006; Goulart et al., 2010; Okubo et al., 2013) and clinical manifestations (Rozenbaum and Gonçalves, 1994; Speed and Dunt, 1995; Ngamskulrungroj et al., 2012b).

Based on the above presented phenotypic and genotypic diversity, the *C. gattii*/*C. neoformans* species complex can be divided into seven haploid and four hybrid genotypes (Table 1) (Boekhout et al., 2001; Bovers et al., 2008a; Hagen et al., 2010a, 2012b; Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009). *Cryptococcus neoformans* var. *grubii* is represented by genotype AFLP1/VNI and the two minor genotypes AFLP1A/VNII (i.e., genotype VNB as described by Litvintseva et al. (2006)) and AFLP1B/VNII (Barreto de Oliveira et al., 2004; Boekhout et al., 2001; Bovers et al., 2008a; Litvintseva et al., 2006; Meyer et al., 2009, 2011). *Cryptococcus neoformans* var. *neoformans* is represented by genotype AFLP2/VNIV, while the hybrid between both varieties fell into genotype AFLP3/VNIII (Boekhout et al., 2001; Li et al., 2012a; Meyer et al., 2009, 2011; Trilles et al., 2003). It is noteworthy that the original isolate reported by Sanfelice (1895) from peach juice in Italy which is maintained as CBS132 turned out to be a serotype AD hybrid (Boekhout et al., 2001; Bovers et al., 2008a; Ikeda et al., 1985, 2000; Kabasawa et al., 1991; Kwon-Chung and Varma, 2006; Li et al., 2012a). Consequently, in the current taxonomic two species

Table 1
Current and proposed species in the *C. gattii*/*C. neoformans* species complex.

Current species name	MLST Clade/ AFLP-genotype[g]	PCR-fingerprinting/RFLP-genotype[h]	Proposed species name
Cryptococcus neoformans var. *grubii*[a]	Clade F, AFLP1 Clade G, AFLP1A/VNB[i] Clade H, AFLP1B	VNI VNII VNII	*Cryptococcus neoformans*
Cryptococcus neoformans var. *neoformans*[b]	Clade I, AFLP2	VNIV	*Cryptococcus deneoformans*
Cryptococcus neoformans intervariety hybrid	AFLP3	VNIII	*Cryptococcus neoformans* × *Cryptococcus deneoformans* hybrid
Cryptococcus gattii[c]	Clade D, AFLP4 Clade C, AFLP5 Clade A, AFLP6 Clade E, AFLP7 Clade B, AFLP10	VGI VGIII VGII VGIV VGIV[j]/VGIIIc[k]	*Cryptococcus gattii* *Cryptococcus bacillisporus* *Cryptococcus deuterogattii* *Cryptococcus tetragattii* *Cryptococcus decagattii*
Cryptococcus neoformans var. *neoformans* × *Cryptococcus gattii* AFLP4/VGI hybrid[d]	AFLP8	–	*Cryptococcus deneoformans* × *Cryptococcus gattii* hybrid
Cryptococcus neoformans var. *grubii* × *Cryptococcus gattii* AFLP4/VGI hybrid[e]	AFLP9	–	*Cryptococcus neoformans* × *Cryptococcus gattii* hybrid
Cryptococcus neoformans var. *grubii* × *Cryptococcus gattii* AFLP6/VGII hybrid[f]	AFLP11	–	*Cryptococcus neoformans* × *Cryptococcus deuterogattii* hybrid

[a] Introduced by Franzot et al. (1999) for serotype A isolates.
[b] Introduced by Franzot et al. (1999) for serotype D isolates.
[c] Introduced by Kwon-Chung et al. (2002) to raise *C. neoformans* var. *gattii* to the species level.
[d] Described by Bovers et al. (2006).
[e] Described by Bovers et al. (2008b).
[f] Described by Aminnejad et al. (2012).
[g] AFLP genotyping nomenclature introduced by Boekhout et al. (2001) and Hagen et al. (2010a, 2012b).
[h] PCR-fingerprinting and RFLP-based genotyping nomenclature as introduced by Meyer et al. (2003).
[i] Introduced nomenclature of a cluster of African isolates (Litvintseva et al., 2006), this was later confirmed to be genotype AFLP1A (Bovers et al., 2008a).
[j] Trilles et al. (2014) based on *URA5*-RFLP.
[k] Springer et al. (2014) based on MLST it was named genotype VGIIIc but compared to ISHAM consensus MLST it was similar to genotype AFLP10.

concept (Kwon-Chung and Varma, 2006) this authentic isolate of *C. neoformans* represents an intervarietal hybrid. *Cryptococcus gattii* can be discerned into five genotypes, with genotypes AFLP4/VGI, AFLP6/VGII and AFLP10/VGIV corresponding with serotype B isolates, while genotypes AFLP5/VGIII and AFLP7/VGIV represent serotype C isolates (Fraser et al., 2005; Hagen et al., 2010a, 2012b; Meyer et al., 2003, 2009, 2011). Recently, interspecies hybrids between *C. gattii* and *C. neoformans* were described, with the hybrid *C. neoformans* var. *neoformans* AFLP2/VNIV × *C. gattii* AFLP4/VGI (serotype BD, genotype AFLP8; Bovers et al., 2006), *C. neoformans* var. *grubii* AFLP1/VNI × *C. gattii* AFLP4/VGI (serotype AB, genotype AFLP9; Bovers et al., 2008b) and *C. neoformans* var. *grubii* AFLP1/VNI × *C. gattii* AFLP6/VGII (serotype AB, genotype AFLP11; Aminnejad et al., 2012).

The status of the various lineages in the *C. gattii*/*C. neoformans* species complex has been debated with two emerging hypotheses, namely (a) the two species concept as supported by Kwon-Chung and Varma (2006) versus (b) the seven species concept as suggested by Bovers et al. (2008a), Meyer et al. (2011) and Ngamskulrungroj et al. (2009). Here we provide strong support for the latter hypothesis by performing phylogenetic analyses of 11 genetic loci of 115 globally collected isolates. The molecular data were used to perform gene tree analyses in a maximum likelihood (ML) and Bayesian (B/MCMC) framework and to generate coalescent-based species trees. We employed a combination of methods to address the species delimitation, using gene tree estimations from single-locus and concatenated data sets, and species tree estimations, including a genealogical species recognition method in which presence of clades in the majority of single-locus genealogies is taken as evidence that these represent distinct lineages (Dettman et al., 2003a,b; Pringle et al., 2005). We also used the coalescent-based general mixed Yule coalescent (GMYC) method (Monaghan et al., 2009; Pons et al., 2006), which aims at locating the nodes that define the transitions between intraspecific (tokogenetic) and interspecific relationships using branch lengths. The method has been used successfully for fungi (Leavitt et al., 2012a,b; Parnmen et al., 2012; Pérez-Ortega et al., 2012; Powell et al., 2011). Additionally, we used an approach that utilizes a species tree estimation method to inform species delimitation decisions by a likelihood ratio test that measures the fit of gene trees within a given species tree (Carstens and Dewey, 2010). For the clinical laboratory, rapid and reliable identification of species is mandatory. The seven haploid lineages could be identified by MALDI-TOF MS which is supported by our investigations in which reference spectra for each of the new species were challenged by more than 400 well characterized isolates of the *C. gattii*/*C. neoformans* species complex. Finally, an overview is presented of the clinical relevance of the new species.

2. Material and methods

2.1. Isolates and culture media

The cryptococcal isolates (Supplementary Table 1) were cultivated on Malt Extract Agar medium (MEA) (Oxoid, Basingstoke, United Kingdom). Cultures were incubated for 2 days at 25 °C. A working collection was maintained on MEA slants for 48 h at 25 °C and at 4 °C, whereas stocks were stored at −80 °C using cryo-vials (Microbank™ system, Pro-Lab Diagnostics, Richmond Hill, ON).

2.2. Genetic analyses

The mating- and serotypes of the *C. neoformans* isolates were determined by using four PCRs that specifically amplify the *STE20***a** and *STE20*α alleles that belong to serotype A and D (Barreto de Oliveira et al., 2004). For *C. gattii* isolates the mating-type was determined as described by Bovers et al. (2009) using two PCRs that specifically amplify the mating-type **a** and α alleles of the *STE12* gene, and serotypes were taken from Hagen et al. (2010a) in which they were analyzed by using an agglutination method (Crypto Check kit; Iatron Labs, Tokyo, Japan).

A selection of 105 cryptococcal isolates from a previous study (Bovers et al., 2008a) was supplemented with ten additional isolates to cover all known genotypes within the species complex as well as to have all available authentic and type strains included (Supplementary Table 1). The 55 *C. gattii* and 60 *C. neoformans* isolates were subjected to amplified fragment length polymorphism (AFLP) fingerprint analysis as previously described (Hagen et al., 2012a), followed by phylogenetic analysis after amplification and sequencing of 11 nuclear loci, i.e., *CAP59*, *GPD1*, IGS, ITS, *LAC1*, *PLB1*, *RPB1*, *RPB2*, *SOD1*, *TEF1* and *URA5*, as described previously (Bovers et al., 2008a; Hagen et al., 2012b; Meyer et al., 2009). A 1000 × bootstrapped Maximum Likelihood phylogenetic analysis was performed using MEGA v5.2 (Tamura et al., 2011) in order to find the most optimal substitution model, namely the Hasegawa–Kishino–Yano model with rates among sites setting 'gamma distributed with invariant sites'.

2.3. Gene tree estimations

Maximum likelihood (ML) analyses of single loci were performed using the program RAxML v7.2.7 (Stamatakis, 2006; Stamatakis and Ott, 2008). The software jModeltest was used to choose the nucleotide substitution model for each single locus (Posada, 2008). The selected models for each locus are shown in Table 2. Bootstrapping was performed based on 2000 replicates with random sequence additions (Felsenstein, 1985). In addition, the concatenated data set was analyzed by using ML with the program RAxML v7.2.7 (Stamatakis, 2006; Stamatakis and Ott, 2008) and a Bayesian approach with the MrBayes 3.1.2 program (Huelsenbeck and Ronquist, 2001). We conducted the concatenated data set analyses using locus-specific partitions as shown in Table 3 and each partition was allowed to have its own parameters (Nylander et al., 2004). No molecular clock was assumed. For the Bayesian analysis a run with 20,000,000 generations starting with a random tree and employing 4 simultaneous chains were executed. Every 100th tree was saved into a file. The first 1,000,000 generations were deleted as the "burn in" of the chain. The program Are We There Yet? (AWTY) was applied to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached (Nylander et al., 2008). Of the remaining trees a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior

Table 2
Alignment length, variable sites for each sampled locus; and locus-specific model of nucleotide substitution identified using the Akaike information criterion in jModeltest.

Locus	Aligned length	# of variable sites	Model selected under AIC
CAP59	560	108	GTR + G
GPD1	553	156	TPM1uf + G
IGS	860	471	TIM3 + G
ITS	463	16	HKY
LAC1	456	146	TPM1uf + G
PLB1	536	136	TPM2uf + G
RPB1	760	120	TIM1 + G
RPB2	645	70	TPM3 + I
SOD1	437	153	TPM2uf + G
*TEF1*α	716	130	TIM2 + G
URA5	639	115	TPM2 + G

Table 3
Presence and ML bootstrap support of monophyletic clades in comparison to concatenated data set.

Data set	Clade A	Placement of Clade B (CBS11687)	Clade C	Clade D	Clade E	Clade F	Clade G	Clade H	Clade I	Number of strongly supported clades (⩾95% ML-BP)
Concatenated data set	100%	Clade B (100%)	100%	100%	100%	100%	97%	100%	100%	15
CAP59	100%	Clade B (95%)	99%	100%	98%	+	–	+	100%	8
GPD1	100%	Clade B (+)	100%	100%	100%	+	–	99%	100%	9
IGS1	99%	Clade B (+)	100%	100%	100%	–	+	+	100%	10
LAC1	100%	Clade B (95%)	95%	100%	100%	–	+	–	100%	10
RPB1	99%	Clade D (+)	99%	+	96%	100%	97%	95%	100%	12
RPB2	96%	Clade B (99%)	100%	99%	99%	–	–	+	100%	9
SOD1	+	Clade D (+)	+	+	+	+	+	+	100%	4
TEF1	100%	Clade B (+)	99%	100%	100%	+	–	99%	100%	9
URA5	+	Clade B (+)	+	97%	100%	95%	+	97%	100%	9
PLB1	100%	Clade B (97%)	97%	100%	100%	+	–	98%	100%	10
ITS	+	–	+	+	+	–[a]	–[a]	–[a]	+	0
Percentage of single locus data sets in which the clade is present	100%	N/A	100%	100%	100%	64%	45%	82%	100%	N/A

+ = Present but support below 95%, – = not present.
[a] Sequences of clades identical and hence clades forming one monophyletic group

probabilities were obtained for each clade. Clades with ML bootstrap support equal or above 70% under ML and posterior probabilities ⩾0.95 were considered as strongly supported. Phylogenetic trees were visualized using the program FigTree (Rambaut, 2009).

2.4. General Mixed Yule Coalescent (GMYC) species delimitation

The general mixed Yule coalescent approach for species delimitation was implemented (Pons et al., 2006). This method aims at detecting shifts in branching rates between intra- and interspecific relationships. Within a likelihood framework it uses chronograms to compare two models: (a) a null model under which the whole sample derives from a single population obeying a coalescent process and (b) an alternative General Mixed Yule Coalescent (GMYC) model. The latter combines equations that separately describe branching patterns within and among distinct lineages following a Yule model including speciation and extinction, whereas intraspecific relationships follow a coalescent process. A likelihood ratio test (LRT) is used to evaluate whether the null model can be significantly rejected. If the GMYC model fits the data significantly better than the null model, the threshold T allows estimating the number of species present in the data set.

Fifty-eight sequences were selected for the GMYC and species tree analyses. As input tree, GMYC requires a fully resolved chronogram, and does not allow polytomies. Since identical sequences potentially inflate the number of recognized putative species, all identical sequences, and those who were so similar that they produced polytomies in the ML tree, were excluded. We made sure to include all strains used for the morphological and physiological description of the species, with the exception of CBS996 (*C. neoformans*; too similar to WM148), CBS10079 (=WM629; *C. deneoformans*; too similar to BD5), and B5748 (*C. tetragattii*; too similar to B5742). The maximum likelihood tree obtained from the concatenated RAxML search was used for this analysis. A chronogram was calculated from the maximum likelihood tree using the penalized likelihood method as implemented in the chronopl command in the Analyses of Phylogenetics and Evolution package (APE) (Paradis et al., 2004; Sanderson, 2002). The GMYC method requires a fully dichotomous chronogram and thus we used multdivtime to convert our chronogram into a fully dichotomous chronogram with internal branches of length zero, where appropriate (Thorne and Kishino, 2002). This modified chronogram was then analyzed using the GMYC package in SPLITS in R v2.10 (http://www.cran.r-project.org/), by using the single and multiple threshold methods. After optimization, we plotted the lineage through time (LTT) plot with the threshold indicated and a chronogram that had the putative species indicated (Nee et al., 1992). Finally, we used the summary command to summarize the output statistics, including the results of the LRT and the indication of the numbers of clusters and entities.

2.5. Estimation of shared and fixed polymorphisms

In order to estimate the isolation of clades, we calculated shared and fixed polymorphisms among clades using the software SITES (Hey and Wakeley, 1997; https://bio.cst.temple.edu/~hey/). Calculations were performed for all pairwise comparisons of species within each of the groups studied.

2.6. Estimation of species trees

A coalescent-based approach was applied, by using the clone corrected 11 loci sequence data set, to test alternative hypotheses of species delimitation, testing the current species delimitation and the delimitation of clades E–I (Fig. 1). The program Species Tree Estimation using Maximum Likelihood (STEM) was used following the protocol outlined previously by Carstens and Dewey (2010) to estimate likelihood scores of alternative species delimitation scenarios among species trees (Kubatko et al., 2009). A set of analyses were performed assuming the present classification of the two species scenario, distinction of all nine clades as species (i.e., the nine species scenario), clades F–H representing a single species (i.e., the seven-species scenario), clades F + G representing one species (i.e., 8a-species scenario), clades F + H representing one species (i.e., 8b-species scenario), and clades G + H representing a single species (i.e., 8c-scenario). Species tree analyses were performed with maximum likelihood gene trees estimated for each locus separately using RAxML v7.2.7 (Stamatakis, 2006; Stamatakis and Ott, 2008). STEM requires fully resolved gene trees, thus polytomies were resolved by using MULTI2DI in the APE package (Paradis et al., 2004). Maximum likelihood scores were evaluated using likelihood-ratio tests (LRTs) to assess statistical significance after correcting for multiple comparisons with a Bonferroni correction.

2.7. Phenotyping assays

Growth patterns on different carbon and nitrogen sources, vitamin requirements, growth at different temperatures and other growth characteristics, such as extracellular starch formation, growth at 50% glucose medium, urease activity and staining with Diazoneum Blue B salt (DBB) were tested according to Kurtzman et al. (2011b).

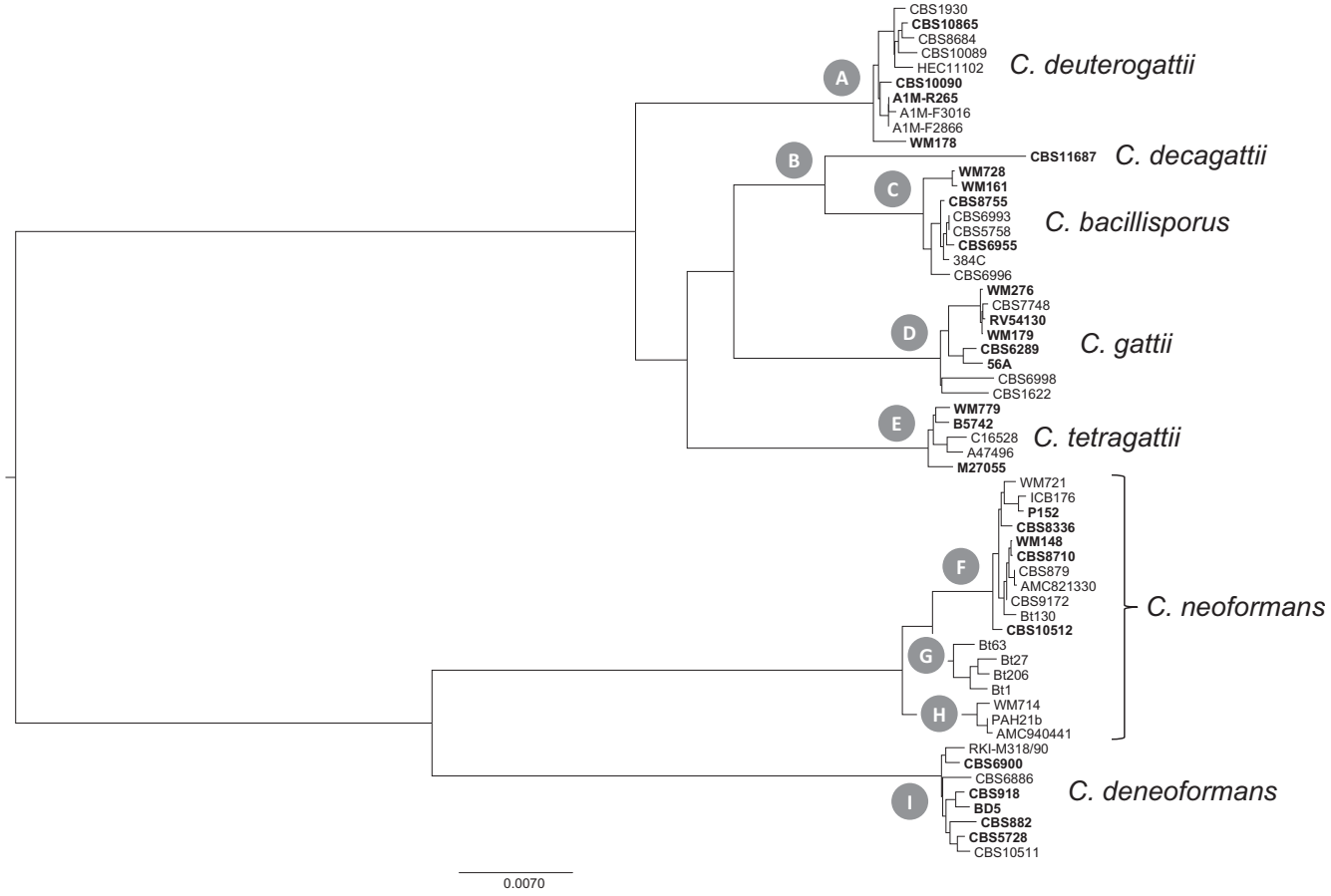

Fig. 1. Diversity in the *C. gattii*/*C. neoformans* species complex inferred from a concatenated data set of 11 loci. The clade letters are discussed in the text, isolates indicated in bold were used for describing the species.

Capsule thickness was measured using Indian ink staining of 20 randomly selected cells per isolate from 1-week-old colonies that were cultured on Littman's ox gall agar (BD, Franklin Lakes, NJ, U.S.A.) at both 25 and 37 °C. The capsule thickness was calculated as the ratio between the diameters of the capsule and the cell.

Cutinase, laccase, lipase, phospholipase and protease activities were investigated in triplicate by plate assays at 25 and 37 °C. Cutinase activity was detected, according to the method described by Dantzig et al. (1986), with *p*-nitrophenyl butyrate (Sigma Aldrich, St. Louis, MO, U.S.A.) as the substrate. The yeast isolates were grown on acetate medium (0.5% yeast extract, 0.5% peptone, 0.5% ammonium acetate, 2.0% agar). After incubation for 10 days at 25 °C, a loop of cells was transferred onto a glass slide and overlaid with freshly prepared 0.026% *p*-nitrophenyl butyrate containing 0.011% Triton X-100 (Sigma Aldrich). Distinct yellow staining was considered as a positive reading.

Lipase activity was detected in triplicate according to the methods described by Sierra (1957) and Middelhoven (1997), using Tween 40, Tween 60 and Tween 80 as substrates. The presence of lipase activity was indicated by a diffuse zone of crystals immersed around the yeast colony and calculated as the ratio between the zone of precipitation and the colony diameter.

Laccase activity was measured in triplicate on L-DOPA and norepinephrine media as described before (Petter et al., 2001) at 25 and 37 °C. The development of brown pigment was scored daily during 10 days, with the following scores: 0 = no melanin production; 1 = center of the colony is dull brown, colony edge has no melanin production; 2 = colony is dull brown; 3 = center of the colony is dark brown, colony edge is dull brown; 4 = colony is dark brown colored; 5 = colony is black colored.

Phospholipase activity was investigated in triplicate using egg yolk plates as described by Vidotto et al. (1996). The presence of phospholipase activity was indicated by the occurrence of a clear zone around the yeast colonies and calculated as the ratio between the zone of enzyme activity and the colony diameter.

Protease activity was assessed in triplicate according to the method described by Braga et al. (1998) using agar plates containing 0.75% casein. The presence of protease activity was indicated by the occurrence of a clear zone around the yeast colonies and calculated as the ratio between zone of enzyme activity and the colony diameter.

The activity of 19 pathogenicity associated enzymes was tested by using API-ZYM (BioMerieux, Marcy l'Etoile, France) (Casal and Linares, 1983). Isolates were cultured on MEA medium and incubated for 48 h at 37 °C after which the API-ZYM test was carried out according to the instructions provided by the manufacturer.

2.8. Antifungal susceptibility testing by the broth microdilution method

The *in vitro* antifungal susceptibility was determined by the broth microdilution method for amphotericin B (Bristol Myers Squibb, Woerden, The Netherlands), 5-fluorocytosine (Valeant Pharmaceuticals, Zoetermeer, The Netherlands), fluconazole and voriconazole (Pfizer Central Research, Sandwich, Kent, United Kingdom), itraconazole (Janssen Cilag, Tilburg, The Netherlands), posaconazole (Schering-Plough Corp., Kenilworth, NJ, U.S.A.), and isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), and minimum inhibiting concentrations calculated according to the recommendations of the National Committee for Clinical

Laboratory Standards (NCCLS M27-3A; Hagen et al., 2010a). Drug-free and sterile controls were included. *Paecilomyces variotii* (ATCC22319) and *Candida krusei* (ATCC6258, CBS573) were used for quality control. Microtiter plates were incubated at 35 °C for 72 h under aerobic conditions.

2.9. Virulence study

Virulence was studied in a murine model as described by Falk et al. (1999) as follows. Yeasts were injected into the tail vein of male albino BALB/c mice (weight, 20 ± 3 g) by administration of a single bolus of a 0.2 ml suspension in PBS. Two inocula were used for each cryptococcal isolate, namely 5×10^5 and 5×10^6 yeast cells per mouse made from a 48 h old culture grown at 30 °C on Sabouraud glucose agar (SGA) (Difco, Detroit, MI, U.S.A.). The yeast concentration was determined by counting with a haemocytometer. Viable count was measured as the number of CFU on SGA plates after 48 h of incubation. Each test group included ten mice. Virulence was based on lethality and determined by recording the number of dead animals in each group daily for 30 days. The virulence was scored between values 4 (high) and 1 (low). Level 4 represented isolates that killed the mice within 5 days (high inoculum) or 10 days (low inoculum); Level 3 – within 12 days (high inoculum) or 20 days (low inoculum); Level 2 and Level 1 represent strains that killed 50% or 10% of the mice within 30 days, respectively.

Cryptococcus neoformans strain H99 was used as a reference. All procedures, care and treatment of mice were in accordance with the principles of humane treatment outlined by the Guide for the Care and Use of Laboratory Animals of the Hebrew University, and were approved by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals (approval number OPRR-A01-5011).

2.10. Matrix-assisted laser desorption ionization-time of flight mass spectrometry identification

The CBS-KNAW in-house made database contained 150 reference Main Spectra (MSPs) of representatives of all genotypic groups of the *C. gattii*/*C. neoformans* species complex (Supplementary Table 2). This set included 74 MSPs from 38 strains of the *C. gattii* complex, 31 MSPs of 17 strains of *C. neoformans* serotype A (currently classified as variety *grubii*), 16 MSPs of 9 strains of *C. neoformans* serotype D (currently classified as variety *neoformans*) and 7 MSPs of 7 hybrid strains including serotype AD, serotype BC and serotype BD. In addition, 16 MSPs of 16 strains of serotype AD hybrid strains grown on SGA were added. Sixty-five strains were cultured on Sabouraud glucose agar (SGA) for 24 h at 30 °C and 69 strains were grown on media that reduce capsule formation, namely SGA + 0.5M NaCl. As no differences were observed in the identification results by using MSPs of *C. gattii* and *C. neoformans* grown on both culture media, for the remainder identifications SGA without salt was used. Ethanol/formic acid protein extraction was done according to the protocol of the manufacturer (Kolecka et al., 2013; Marklein et al., 2009). The test set of 423 *Cryptococcus* isolates included representatives of the *C. neoformans* ($n = 225$) and the *C. gattii* complexes ($n = 145$), and serotype AD, serotype AB and BD isolates ($n = 53$). All these isolates were previously characterized by using a combination of molecular techniques, including AFLP fingerprinting, multi-locus sequence typing (MLST) and mating- and serotyping PCRs (Bovers et al., 2006, 2008a,b, 2009; Hagen et al., 2010a, 2012a,b, 2013; Khayhan et al., 2013; Pan et al., 2012). The isolates used to test the MSPs present in the database were measured in duplicates and identified using a 96-spot polished steel target plate (Bruker Daltonics) as described before (Kolecka et al., 2013). Bacterial Test Standard solution (Bruker Daltonics) was included as a positive control in all test series. MALDI-TOF MS identification results were achieved automatically as the log-score values defined as the 'secure species identification' (>2.0), 'secure genus identification' (1.7–2.0) and 'no reliable identification' (<1.7, NRI). The identification was considered correct if at least one spot from the duplicates gave a reliable identification with score >1.7.

3. Results

3.1. AFLP and multi-gene sequence analysis

AFLP fingerprinting separated the isolates in clusters that were fully concordant with previously observed genotypic groups (Barreto de Oliveira et al., 2004; Boekhout et al., 2001; Bovers et al., 2006, 2008a,b; Hagen et al., 2010a, 2012a,b). The current *C. neoformans* variety *grubii* was represented by three subclusters, namely AFLP1/VNI ($n = 25$), AFLP1A/VNII/VNB ($n = 7$) and AFLP1B/VNII ($n = 8$), whereas *C. neoformans* var. *neoformans* as interpreted hitherto was represented by one cluster, namely AFLP2/VNIV ($n = 20$). *Cryptococcus gattii* fell apart in five different clusters, named AFLP4/VGI ($n = 18$), AFLP5/VGIII ($n = 10$), AFLP6/VGII ($n = 19$), AFLP7/VGIV ($n = 6$) and AFLP10/VGIV ($n = 2$).

Subsequent multi-gene sequence analysis using 11 nuclear loci resulted in a similar clustering (Figs. 2 and 3; Table 1) as observed with the AFLP genotyping and was in agreement with previously obtained results (Bovers et al., 2008a; Diaz et al., 2000, 2005; Hagen et al., 2010a, 2012b; Meyer et al., 1999, 2003, 2009). When all eleven loci were analyzed individually, the single gene trees were concordant between the various loci (Supplementary Figs. 1 and 2).

3.2. Gene tree estimations and species delimitations

The number of nucleotide positions and variable sites for each locus are given in Table 2 together with optimal evolutionary models as determined using AIC. The resulting clades, their genotypic affiliation and new taxonomy ranks are given in Table 1. Single locus ML analyses revealed highly congruent tree topologies with seven clades and one singleton: five (clades A–E, G) were strongly supported in all single locus analyses (Table 3), one clade was present in all but two analyses (clade H), and two clades were present in 45% (clade G) and 64% (clade F) of the single locus analyses, respectively. In addition, one strain (CBS11687) did cluster with either clade C or E in the single locus analyses (data not shown). In the concatenated analyses, the ML and Bayesian searches revealed almost identical trees with strong support for all clades identified in the single locus analyses (Table 3). In the combined analysis sample CBS11687 clustered strongly supported with clade C but with a long branch leading to it.

The coalescence-based single threshold General Mixed Yule Coalescent (GMYC) identified eight putative species (clades A–I) with the concatenated data set, and between six and eight in the single locus analyses (Table 4). In the multiple threshold method, ten putative species were found with the concatenated data set, and between seven and eleven in the single locus analyses. Except for the *URA5* data set, single and multiple threshold analyses did not reveal significantly different likelihood values and, hence, in those cases the simpler single threshold method was preferred. In the *URA5* data set, the single threshold result was significantly better than the multiple threshold result and, consequently, for all analyses the single threshold result was preferred.

The protocol by Carstens and Dewey (2010) was applied to generate species trees from a given set of gene trees under different species delimitation scenarios. By using the same set of gene trees, scenarios were compared that included a nine-species, three different eight-species scenarios, a seven-species scenario, and the

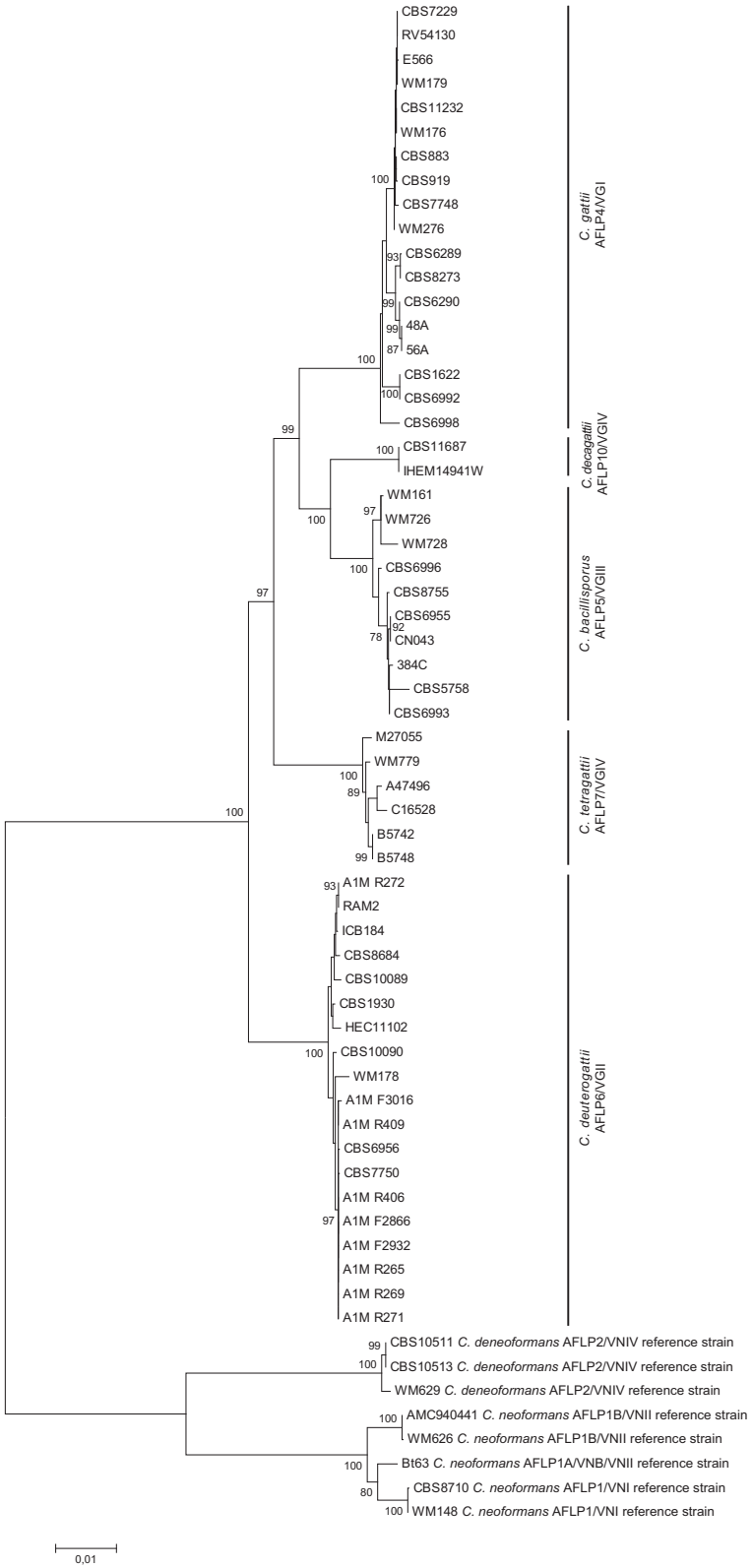

Fig. 2. Concatenated loci phylogenetic bootstrapped maximum likelihood analysis of *C. gattii* species complex isolates by using *C. neoformans* species complex isolates as an outgroup.

current two-species scenario, as explained under Section 2. An information-theoretic approach that accommodates for number of parameters strongly supported a seven-species scenario with a Log Likelihood value of 19054.752 (Table 5) over the set of other species delimitation scenarios (Bonferroni corrected *P*-values <0.001 in all cases). The current two-species classification received the worst likelihood scores of 30090.767 (Table 5). Thus based on the above given considerations there is strong evidence for a seven species concept in the *C. gattii*/*C. neoformans* species complex. See Figs. 1–4.

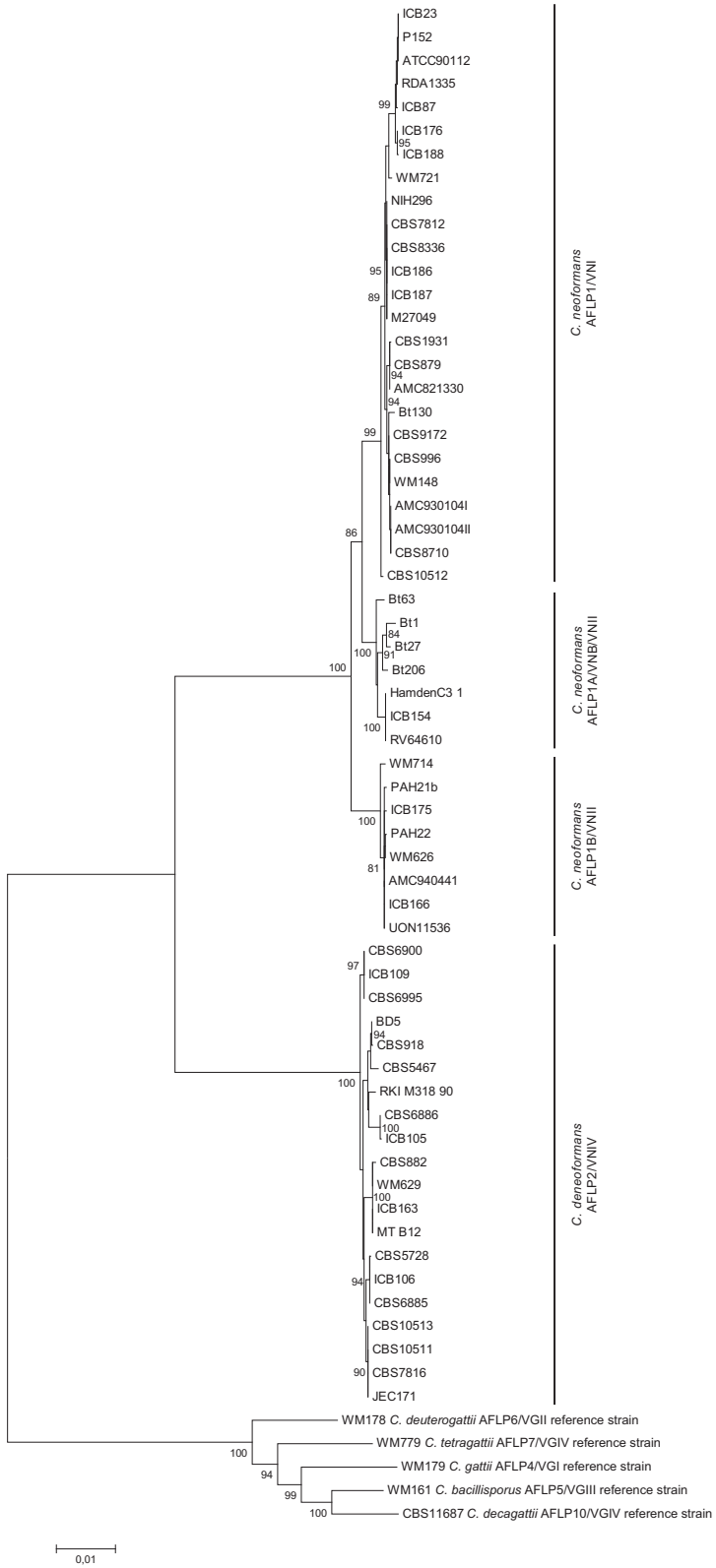

Fig. 3. Phylogenetic bootstrapped maximum likelihood analysis of *C. neoformans* species complex isolates by using *C. gattii* species complex isolates as an outgroup.

3.3. Phenotypic characteristics

The physiological growth profiles by using a large diversity of carbon and nitrogen compounds did not differ widely among the species (for the formal taxonomy see below under Taxonomy) and interspecies hybrids as many carbon sources were utilized by all species (Table 6; Supplementary Fig. 3; Supplementary Table 3). D-Galactonate was not utilized by isolates of *C. deneoformans* and malic acid was used by most isolates of the *C. gattii* species complex, and less by those from *C. neoformans*. Most isolates of *C. neoformans* and *C. deneoformans* grew on propane-1,2-diol, whereas the serotype AD hybrids, representatives of the *C. gattii*

Table 4
Results of species delimitation using the GMYC method for the concatenated and the single locus data sets including single and multiple thresholds.

Data set	$L(0)$	$L(\text{GMYC}_{\text{single}})$	P	Number of putative species	$L(\text{GMYC}_{\text{multiple}})$	P	Number of putative species	Difference between single and multiple threshold p
Concatenated data set	467.894	474.510	0.0042*	8	475.101	0.0061*	10	0.758^{NS}
CAP59	237.397	240.112	0.143^{NS}	6	240.929	0.216^{NS}	7	0.950^{NS}
GPD1	242.255	246.182	0.049*	8	246.948	0.095^{NS}	8	0.957^{NS}
IGS	245.908	265.983	⩽0.001	7	267.906	⩽0.001	7	0.065^{NS}
LAC1	248.466	250.969	0.171^{NS}	8	251.705	0.166^{NS}	7	0.689^{NS}
RPB1	246.686	252.041	0.013*	8	253.052	0.026*	9	0.918^{NS}
RPB2	244.045	249.083	0.018*	7	249.739	0.044*	8	0.971^{NS}
SOD1	243.615	248.551	0.019*	8	248.797	0.110^{NS}	8	0.999^{NS}
TEF1α	248.947	251.275	0.199^{NS}	7	252.669	0.188^{NS}	11	0.835^{NS}
URA5	246.741	259.301	⩽0.001	6	252.124	0.029*	9	0.0024*
PLB1	240.696	242.664	0.268^{NS}	7	243.696	0.389^{NS}	7	0.984^{NS}
ITS	219.457	226.953	0.002*	7	224.921	0.053^{NS}	7	0.668^{NS}

NS = not significant.
* Significant difference at $p < 0.05$.

Table 5
Likelihood scores for STEM analysis of species delimitation scenarios.

Scenario	$-\ln L$	k	$2(\Delta - \ln L)$	Bonferroni corrected P
7-species	19054.752	8	0	
8a-species	20823.570	9	3537.636	<0.001
8b-species	22535.729	9	6961.954	<0.001
9-species	22536.085	10	6962.666	<0.001
8c-species	23413.632	9	8717.76	<0.001
2-species	30090.767	3	22072.03	<0.001

k = number of parameters.

species complex and the *C. gattii* × *C. neoformans* and *C. gattii* × *C. deneoformans* hybrids did not. With respect to the growth on N-compounds, creatinine and L-lysine were assimilated by all isolates. Creatine was not utilized by (most) isolates of *C. neoformans* and *C. deneoformans*, but allowed growth of most isolates of *C. deutorogattii* and *C. tetragattii*. Ethylamine and D-proline were not utilized by isolates of *C. neoformans* and *C. deneoformans*, but allowed growth of most of the isolates of the *C. gattii* species complex (Table 6, Supplementary Table 3). No growth occurred with cadaverine (except a few *C. deneoformans* and the serotype AD hybrid isolates), glucosamine, imidazole (note that *C. deuterogattii* and *C. tetragattii* isolates had a ring of growth at some distance from the well), nitrate, nitrite, and D-tryptophan. All isolates of all species utilized extracellular starch, hydrolyzed urea, showed a positive DBB staining and required vitamins for growth. Most isolates were able to grow on 50% glucose, but (most) not on 60% glucose. All isolates were able to grow at 25, 30, 35 and 37 °C (note that isolate CBS5467 (*C. deneoformans*) and CBS919 (*C. gattii*) showed variable growth at the latter temperature), growth was variable at 40 °C but absent in isolates of *C. bacillisporus*, and no growth occurred at 42 °C.

Although melanisation rates were quite variable, the highest values were seen in serotype AD hybrids as all isolates had black colored colonies after 24 h at both 25 °C and 37 °C, and was found to be slower in isolates of *C. neoformans*, *C. bacillisporus* and *C. deuterogattii*, whereas melanisation rates were lowest in isolates of *C. deneoformans* and *C. gattii* (Suppl. Table 3). Isolates of *C. deuterogattii* showed higher melanisation rates at 25 °C on norepinephrine media (Supplementary Fig. 3).

Phospholipase activity showed a variable result for almost all species. Several isolates showed a somewhat greater phospholipase activity at 25 °C. Protease activity was low and variable among the isolates of the various species.

Thickest capsules were present in isolates of *C. gattii* followed by those of *C. neoformans*, *C. deneoformans*, *C. bacillisporus* and *C. deuterogattii* (Supplementary Table 3). Isolates of the serotype AD hybrids had the thinnest capsules and the driest colonies as well.

Cutinase activity was not observed in any species (except the *gattii* × *neoformans* hybrid) at 25 °C, but occurred at 37 °C in most isolates of *C. neoformans*, *C. deneoformans* and *C. bacillisporus*. Lipase activities, tested using Tweens 40, 60 and 80 plates, showed variable reactions among the various species. Activity with Tween 40 was higher at 25 °C than at 37 °C. Strains CBS6996 (*C. bacillisporus*) and isolates of *C. tetragattii* showed the highest activities. No activities were seen for the following enzymes (except for a few isolates): lipase C14, valine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, *n*-acetyl-β-glucoaminidase, α-mannosydase and α-fucosidase. Activities of

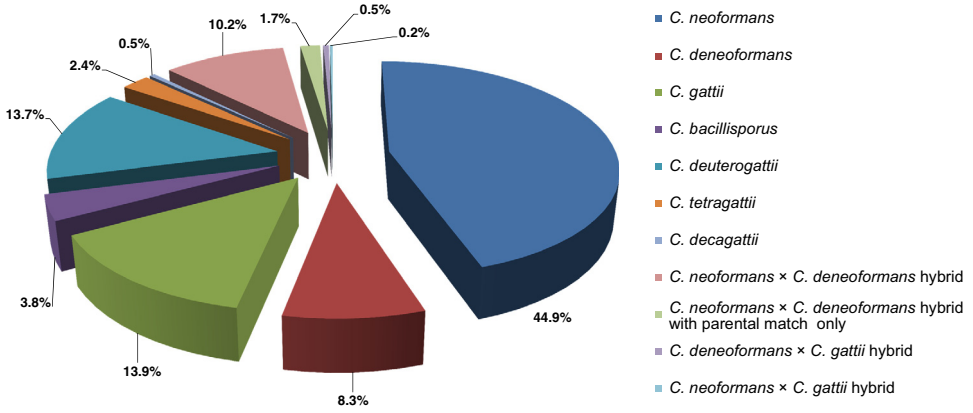

Fig. 4. Diagram showing results of identification of test set of all species and interspecies hybrids by MALDI-TOF MS.

Table 6
Salient phenotypic data for species in the *C. gattii*/*C. neoformans* species complex.

Phenotypes	Species						
	C. neoformans	*C. deneoformans*	*C. gattii*	*C. bacillisporus*	*C. deuterogattii*	*C. tetragattii*	*C. decagattii*
D-Lactate	+	+	+	+	− (+)	+	+
Propane-1,2-diol	+	v	−/w	−/w	−	−/w	+
D-Galactonate	+ (−)	−	+	v	+ (−)	+	?
Malic acid	v	v	+	v	+	+	?
Inulin	v	− (+)	+ (−)	+	+	+	+
Ethylamine	− (w)	− (w)	+	+	+	+	+
D-Proline	−	−	+	+	v	+	+
Creatine	−	−	v	v	v	v	−
CGB medium	−	−	+	+	+	+	+
Esterase C4	+	+	w	w	w	w	w
ECV amphotericin B (μg/ml)[a]	0.5[d]/1	1	0.5	1	0.5[e]/1	1	1[f]
ECV 5-fluorocytosine (μg/ml)[a]	8[d]/16	16	4	4	16	4	4[f]
ECV fluconazole (μg/ml)[b]	8[d]/16	16	8	8	8[e]/32	16	16[f]
ECV itraconazole (μg/ml)[b]	0.25[d]/0.5	0.5	0.5	0.5	0.5	1	1[f]
ECV posaconazole (μg/ml)[b]	0.25	0.25	0.5	0.5	0.5	0.5	0.5[f]
ECV voriconazole (μg/ml)[b]	0.25	0.12	0.5	0.25	0.25	0.25	0.25[f]
ECV isavuconazole (μg/ml)[c]	0.06/0.12[d]	0.06	0.25	0.25	0.25	0.25	0.25[f]

Phenotypic scoring: + = growth; − = no growth; v = variable growth; w = weak growth; value between brackets indicate deviation from main growth pattern.
[a] Epidemiological cut-off values (ECVs) as determined by Espinel-Ingroff et al., 2012a.
[b] Espinel-Ingroff et al., 2012b.
[c] Espinel-Ingroff et al., 2015.
[d] ECV for *C. neoformans* genotype AFLP1/VNI.
[e] ECV for *C. deuterogattii* genotype AFLP6A/VGIIa.
[f] Due to the low number of *C. decagattii* isolates, the general ECVs for the *C. gattii* species complex, or when applicable those for *C. tetragattii* were applied (*C. decagattii* cannot be discerned from *C. tetragattii* by low-discriminatory fingerprint techniques).

esterase C4, esterase lipase C8, leucine arylamidase, acid phosphatase, α-glucosidase and β-glucosidase were highest in strains of the *C. neoformans* complex (Supplementary Fig. 3; Supplementary Table 3).

3.4. Antifungal susceptibility testing

All tested isolates were susceptible for amphotericin B and had MICs equal or lower than the defined epidemiological cut-off values (ECVs) of 0.5 and 1 μg/ml (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2012a). For 5-fluorocytosine, MICs were available for isolates of the *C. gattii* species complex and were observed to be lower, equal and one dilution-step higher than the defined ECV of 4 μg/ml for *C. tetragattii*, and below the ECV of 16 μg/ml for *C. deutrogattii*. When the general *C. gattii* species complex ECV for 5-fluorocytosine was considered (16 μg/ml) than all isolates had MICs lower than the ECV of 16 μg/ml (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2012a).

Cryptococcus neoformans isolates had MICs for fluconazole equal or lower than the ECV of 8 μg/ml, none of the *C. deneoformans* isolates had a MIC for fluconazole that were higher than the defined ECV of 16 μg/ml for non-typed *C. neoformans* species complex isolates (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2012b). Similarly, none of the intervariety hybrid *C. neoformans* × *C. deneoformans* and *C. gattii* species complex isolates had fluconazole MICs that were higher than the proposed ECVs (Supplementary Table 3; Espinel-Ingroff et al., 2012b). MICs for itraconazole and posaconazole were for all tested isolates equal or below the proposed ECVs (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2012b). Nearly all isolates had MICs for voriconazole that were equal or below the proposed ECV, however, some *C. deuterogattii* isolates had MICs that were 1 μg/ml and were higher than the proposed ECV of 0.25 μg/ml (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2012b). All tested *C. neoformans* isolates, except one, had MICs that were below the recently proposed ECV of 0.12 μg/ml for isavuconazole. For *C. deneoformans* and the intervariety *C. neoformans* × *C. deneoformans* isolates no ECV was established, but the tested isolates had MICs that were equal or below the ECV of *C. neoformans* (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2015). The proposed isavuconazole ECV of 0.25 μg/ml was established for isolates that belongs to the *C. gattii* species complex. In general the MICs observed were below this ECV but some *C. gattii* and *C. deuterogattii* had MICs that were higher (Table 6, Supplementary Table 3; Espinel-Ingroff et al., 2015).

3.5. Virulence assays

Most of the isolates tested for virulence showed low to moderate virulence using both high and low inocula. Three isolates showed high virulence, namely CBS5467 of *C. deneoformans* (genotype AFLP2) from milk of a mastitic cow), a *C. neoformans* × *C. deneoformans* hybrid isolate NY-J40 (genotype AFLP3) from an AIDS patient, and CBS6993 of *C. bacillisporus* (genotype AFLP5) isolated from a non-AIDS patient.

3.6. Identification of genotypic groups by MALDI-TOF MS

By using the CBS-KNAW in-house database together with the Bruker BDAL database (3995 MSPs, v3.1.2.0) all 423 isolates (100%) of the *C. gattii*/*C. neoformans* species complex were identified by MALDI-TOF MS at the species level (Fig. 4). By using duplicates, 322 isolates (76.1%) were identified with scores >2.0 on both spots, 98 strains (23.2%) had scores >2.0 only on one spot from duplicates, and three strains (0.7%) gained scores (1.7–2.0) on both spots. Four attempts were needed to accomplish measurements of all strains enrolled for testing by MALDI-TOF MS.

The identifications generated by MALDI-TOF MS were further evaluated for the correct genus and species recognition based on the previous molecular and genetic analyses as well as for the concordance with one of the seven species and the intervariety and interspecies hybrids. Overall, a correct identification at the species and AFLP genotype level was obtained for 415 (98.1%) out of 423 isolates. Straightforward identification and AFLP genotype match gained all isolates of *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii* and *C. decagattii* and the *C. neoformans* × *C. gattii* and

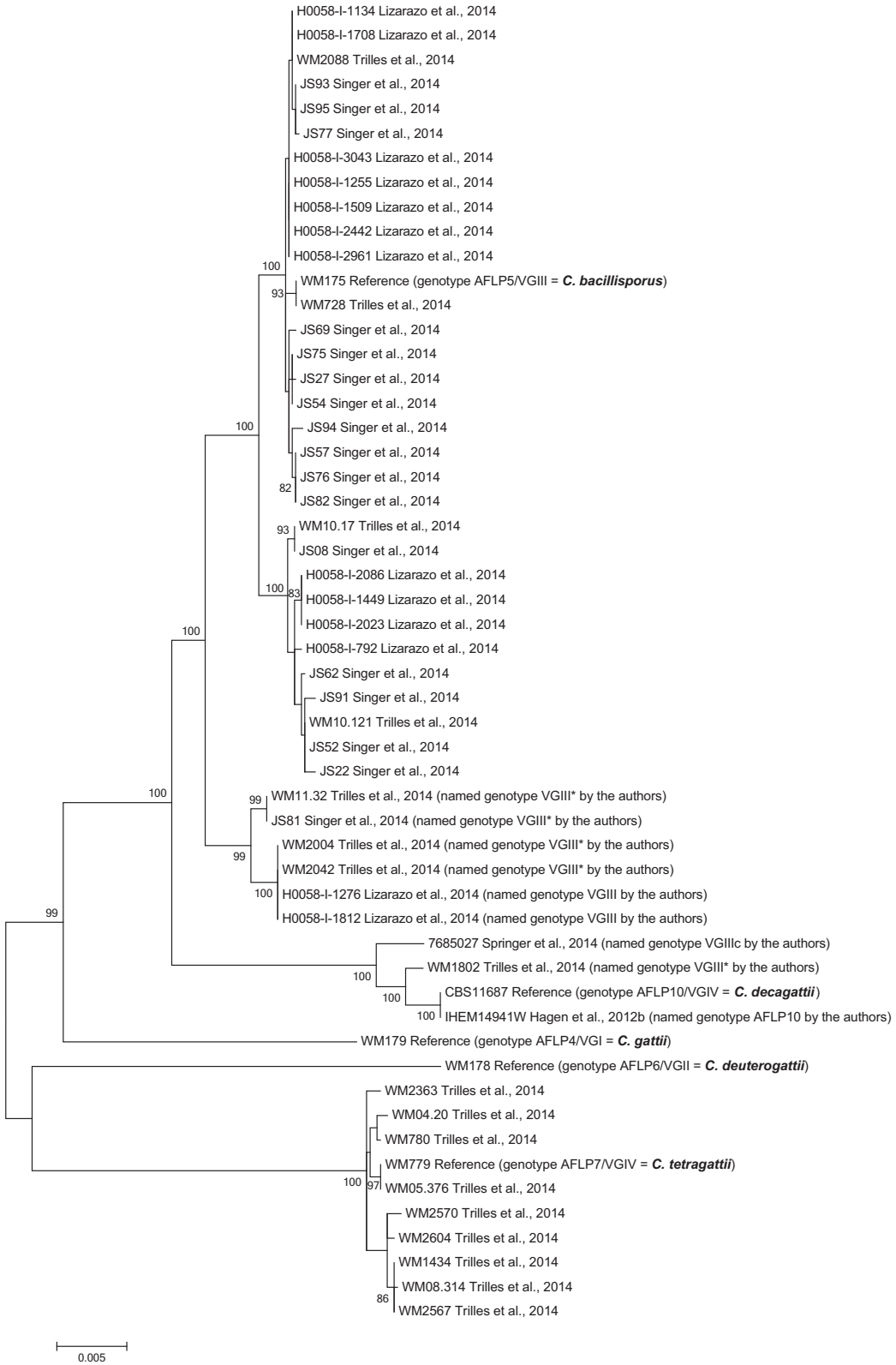

Fig. 5. Re-analysis of available MLST-data from isolates that fell into putative *C. bacillisporus* and *C. decagattii* clusters.

C. deneoformans × *C. gattii* hybrids. Fifty-eight from the 59 *C. gattii* isolates were correctly identified on both spots, one isolate (4–308), however, gained a *C. gattii* species complex identification and matched with *C. gattii* on one spot and *C. deuterogattii* on the other. The recognition of *C. deneoformans* was correct for 34 of the 35 (97.1%) isolates, and one isolate (CBS7814) gained an atypical hybrid match with serotype AD hybrids and a 'No Peaks Found'-score. A correct recognition of the serotype AD hybrids resulted for 26 (52%) of 50 strains, whereas the remaining 24 isolates gained either a 'parent/parent' or a 'hybrid/parent' match from duplicates. For 13 isolates a serotype AD match was detected on one spot and *C. neoformans* on the second. For 4 isolates a

correct hybrid match was found on one spot and *C. neoformans* on the second. Six isolates matched with *C. neoformans* on both spots and one strain with *C. deneoformans* on both spots.

4. Discussion

The taxonomy of the *Cryptococcus gattii*/*C. neoformans* species complex has been debated since the extensive genetic and biochemical diversity within the complex became clear (Kwon-Chung and Varma, 2006). At the 6th and 8th International Conferences on *Cryptococcus* and Cryptococcosis (ICCC6 and 8) held in 2005 in Boston and 2011 in Charleston, lively debates on the matter were held (Coenjaerts, 2006; Del Poeta and Casadevall, 2012; Del Poeta et al., 2005). Before the ICCC6 meeting was held, a nomenclature paper settled the taxonomic position of *C. gattii* (Kwon-Chung et al., 2002), and this decision was readily adopted by the research and clinical communities. However, the discussion on the taxonomic status of the genotypic groups as recognized in both *C. neoformans* and *C. gattii* continued (Bovers et al., 2008a; Hagen et al., 2010a, 2012b; Kwon-Chung and Varma, 2006; Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009). Importantly, fully concordant results (Table 1) were obtained by many molecular taxonomic investigations irrespective whether they were based on enzyme typing (Brandt et al., 1993), DNA fragment analysis (e.g., AFLP, RFLP, PCR-fingerprinting), sequence analysis of a number of genes (Bovers et al., 2008a; Byrnes et al., 2010, 2011; Findley et al., 2009; Fraser et al., 2005; Hagen et al., 2012b, 2013; Litvintseva and Mitchell, 2012; Litvintseva et al., 2006; Meyer et al., 2011; Ngamskulrungroj et al., 2009; Springer et al., 2014; Sugita et al., 2001), and comparative genomics data (Billmyre et al., 2014; Cuomo and Litvintseva, 2014; D'Souza et al., 2011; Engelthaler et al., 2014; Fisher, 2014; Heitman, 2014; Meyer et al., 2011; Sun et al., 2013; Voelz et al., 2013).

4.1. Taxonomic and phylogenetic concepts

Here, we used genealogical (Taylor et al., 2000) and coalescent-based (Fujita et al., 2012) species delimitation methods based on phylogenetic trees of 11 genomic loci. Both genealogical and coalescent-based analyses demonstrated that the current two species classification does not reflect the phylogenetic relationships within the *C. gattii*/*C. neoformans* species complex, but that more distinct lineages exist within the group. All methods strongly supported seven clades (labeled A–I in Fig. 1; Tables 3–5) to be distinct lineages, i.e., species. We consider the congruence of different methods that rely on independent analysis, i.e., monophyly and support in single locus analyses in genealogical approaches, differences in branch lengths in GMYC, and likelihood values of species trees, as a strong indication that those seven lineages represent species. The results for clades F and G differed between the analyses and we have chosen here a conservative approach and consider them genotypes within *C. neoformans*, since we agree with Carstens et al. (2013) that it is better to fail to delimit species than to falsely delimit entities that do not represent distinct evolutionary lineages and therefore a conservative approach was chosen that potentially underestimates the diversity in the group. Clade H was supported as a distinct lineage, while the genealogical and species tree approaches did not support them as distinct, the GMYC method identified them as distinct putative species. However, the GMYC has been shown to overestimate the number of species in specific cases, although it has mostly been shown to be a robust tool for species delimitation (Fujisawa and Barraclough, 2013; Hamilton et al., 2014; Reid and Carstens, 2012; Talavera et al., 2013). Furthermore, isolates of genotypes AFLP1A/VNB/VNII (clade G) and AFLP1/VNI (clade F) of *C. neoformans* (serotype A) were found to recombine (Litvintseva et al., 2003, 2006) thus giving further evidence to consider them as a panmictic group.

Many species concepts are in use in biology, but in mycology the phenetic, biological and phylogenetic species concepts have been most widely used. When reviewing the available literature on the taxonomy of the *C. gattii*/*C. neoformans* species complex it occurred to us that most published data are in agreement, or at least not in conflict, with the recognition of the current main genotypic groups as species. Recently, the species concept as applied in a number of eukaryotic pathogens, including *Cryptococcus* spp., was discussed using the concept of Predominant Clonal Evolution (PCE) (Tibayrenc and Ayala, 2014). Hallmarks of PCE are highly significant linkage disequilibrium and near-clading ('clonal near-clades'). The latter was described as 'stable phylogenetic clustering clouded by occasional recombination' and according to these authors both occur in *Cryptococcus* spp. (Tibayrenc and Ayala, 2014). As far as we know no recombination has been detected between the major genotypic groups in the *C. gattii*/*C. neoformans* species complex, although this may be blurred by the occurrence of interspecies diploid or aneuploid hybrids that are heterozygous and genetically not very stable. Reversals to (near) haploid stages cannot be ruled out and selection of one of the orthologs from the hybrid progeny may be interpreted as recombination. In our opinion the presence of these hybrids strongly suggests post-zygotic isolation mechanisms that hinder regular meiosis (see also below). We do not favor PCE in the case of the *C. gattii*/*C. neoformans* species complex as the species are characterized by stable multigene data and other genotypic methods such as PCR-fingerprinting and AFLP, they can be identified phenotypically by differences in the proteome (MALDI-TOF MS), and they show differences in disease symptoms and susceptibility to antifungals. Moreover, emerging whole genome analyses support their genetic separation as well. The concept of PCE seems valid at the population level within the species that we recognize as recombination may occur (e.g., in *C. neoformans*, *C. deuterogattii*), and clonal expansions may result in the relative dominance of some subgenotypes and α mating-types, e.g., in *C. neoformans*, *C. bacillisporus* and *C. deuterogattii*.

Kwon-Chung and Varma (2006) strongly favored a two species concept in the complex. This was based on the notion that many phenotypic differences have been documented to occur between isolates of the *C. neoformans* and *C. gattii* species complexes. We do not dispute this, but, on the contrary, novel technologies, such as MALDI-TOF MS, distinguished all major genotypes (Firacative et al., 2012; Hagen et al., 2011; Posteraro et al., 2012; current study). All molecular taxonomic studies separated the major genotypes (see above) in the complex, but in some studies alleles of serotype AD hybrid isolates clustered with either serotype A or D isolates (see e.g., Diaz et al., 2005 and the discussion on this topic in Kwon-Chung and Varma, 2006). These observations are, however, a consequence of the diploid or aneuploid nature of hybrid genomes that may have two alleles for all or many genes, one representing the serotype A allele and the other the serotype D allele. In case these alleles have not been cloned before sequencing, which was the case in the Diaz et al. (2005) study, an erratic phylogenetic pattern may occur. One may interpret this as a lack of concordance between genotypes and serotypes as was done by Kwon-Chung and Varma (2006), but in our opinion this is a consequence of the hybrid nature of such genomes and the methodological limitations of studies when genes are directly sequenced from PCR-amplicons.

4.2. Comparative genomics and sequence divergence

The amount of sequence divergence between single copy orthologs in the genomes of strain H99 (serotype A, genotype AFLP1/VNI) and JEC21 (serotype D, genotype AFLP2/VNIV) was on

average 7%, and between the genomes of both these strains and *C. gattii* WM276 (serotype B, genotype AFLP4/VGI) this was 11% (Janbon et al., 2014). In another study the divergence between the genomes of B3501A (serotype D, genotype AFLP2/VNIV) and *C. gattii* WM276 (serotype B, genotype AFLP4/VGI) was found to be 13% and between the genomes of B3501A and *C. gattii* A1M-R265 this was 14.5% (serotype B, AFLP6A/VGIIa) (D'Souza et al., 2011). The genomes of the two serotype B strains WM276 and A1M-R265 (D'Souza et al., 2011), as well as those of *C. neoformans* var. *neoformans* JEC21 (serotype D, genotype AFLP2/VNIV) and *C. neoformans* var. *grubii* H99 (serotype A, genotype AFLP1/VNI) (Janbon et al., 2014) were found to be largely co-linear, whereas between the genomes of the two serotype B strains and both the serotype A and serotype D strains many fixed and unfixed rearrangements were observed (D'Souza et al., 2011; Janbon et al., 2014; Sun and Xu, 2009). Phylogenomic analysis of 5171 single gene orthologs indicated that the time since divergence of serotype A versus both serotype B strains was found to be ~34 million years (myr) ago, whereas divergence between genotype AFLP4/VGI and AFLP6/VGII was 7.6% corresponding to ~12.4 myr (D'Souza et al., 2011). Divergence between serotype A (H99, genotype AFLP1/VNI) and serotype D (JEC21/B3501, genotype AFLP2/VNIV) was estimated ~20 myr (time as indicated in Fig. 2 in D'Souza et al., 2011). Another study suggested that the *C. neoformans* and *C. gattii* complexes separated 80 myr, but with a broad range of 16–160 myr. A population genomics study of the *C. gattii* species complex, focusing on North American outbreak isolates, studied the genomes of 118 isolates and found that the four major genotypes, i.e., AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, and AFLP7/VGIV, clustered separately with more than 500,000 SNPs occurring between AFLP6/VGII and the other genotypes (Engelthaler et al., 2014). Patterns of intron evolution also separated the *C. gattii* and *C. neoformans* species complexes (Croll and McDonald, 2012). Genomes of AFLP4/VGI, AFLP5/VGIII and AFLP6/VGII, AFLP1/VNI and AFLP2/VNIV showed distinct patterns of intron presence/absence. Nineteen isolates of AFLP6/VGII had the same intron pattern, as did two isolates of AFLP4/VGI, and two isolates of AFLP2/VNIV (Croll and McDonald, 2012).

Multigene phylogenetic analyses yielded somewhat different divergence times (Meyer et al., 2011; Ngamskulrungroj et al., 2009; Xu et al., 2000). Concatenated sequences of the mtLrRNA, ITS, and *URA5* and *LAC* genes (Xu et al., 2000) and the *ACT1*, *IDE1*, *PLB1*, and *URA5* genes (Meyer et al., 2011) suggested that the *C. neoformans* and the *C. gattii* species complexes separated 37 or 49 myr based on the two data sets, respectively; the two current varieties in *C. neoformans* separated 18.5 and 24.5 myr, respectively, the four included lineages within *C. gattii* became distinct between 2.6–9.5 and 8.5–12.5 myr, and the AFLP1/VNI and AFLP1A/VNB/VNII lineages of serotype A separated 4.7 myr. Introgressions have been observed to occur between some of the genotypic groups in the *C. gattii*/*C. neoformans* that we propose to represent species. For instance, a 14-gene region of approximately 40 kb in size was found to be horizontally transferred from a strain with serotype A background to one with a serotype D background (Kavanaugh et al., 2006). In our opinion these comparative gene phylogenies and genomics data strongly suggest that the current major genotypes of the *C. gattii*/*C. neoformans* species complex should be interpreted as species.

4.3. Phenotyping

Because a considerable number of phenotypic and genotypic differences has been observed to occur between the *C. neoformans* and *C. gattii* species complexes, we wondered whether they agree with the recognized genotypes and if they differ in phenotypic aspects. Therefore, we investigated whether isolates belonging to the various species differed with respect to virulence related factors, such as capsule thickness, rate of melanin formation, lipase, phospholipase and protease activity, as well as nutritional requirements, and antifungal susceptibility to clinically used antifungal compounds. Several phenotypic data separated the *C. neoformans* species complex from the *C. gattii* species complex, e.g., the CGB reaction, some stronger enzyme activities, growth on ethylamine, and D-proline, and to some extent malic acid. Furthermore, *C. deneoformans* (AFLP2/VNIV) was able to grow on D-galactose, which was absent in the remainder. Because the natural niche of *C. neoformans* and *C. gattii* species complexes seem to be on trees and tree-related habitats (Chowdhary et al., 2011, 2012a,b, 2013; Colom et al., 2012; Hagen and Boekhout, 2010; Kidd et al., 2007; Lazéra et al., 1998; Springer and Chaturvedi, 2010), we tested a number of enzymes, such as cutinase, laccase and lipase that play a role in the degradation of plant material. When compared to an older study (Schmeding et al., 1984) in which the nutritional requirements of carbon and nitrogen compounds of 79 strains was analyzed a number of differences became apparent. For instance, most if not all strains in our study utilized citric acid, creatinine, erythritol, inulin, sorbose and succinic acid. In agreement with other authors (Dufait et al., 1987; Kwon-Chung and Varma, 2006) most strains of the *C. gattii* species complex were able to utilize D-proline and all were CGB positive. Furthermore, they were also able to grow with ethylamine. Contrary to most other species, strains of *C. deneoformans* (genotypic group AFLP2/VNIV) did not utilize glycerol. Ngamskulrungroj et al. (2012a) observed that isolates of the *C. gattii* species complex were able to grow with D-alanine, L-phenylalanine and L-tryptophane, but not isolates of the *C. neoformans* species complex. Unlike *C. neoformans*, isolates of *C. deneoformans* (genotypic group AFLP2/VNIV) grew poorly with L-phenylalanine and L-tryptophan (Ngamskulrungroj et al., 2012a). Contrary to L-tryptophan we observed that D-tryptophan could not be utilized by any member of the *C. gattii*/*C. neoformans* species complex. Our data suggest differences in the activities of a number of extracellular enzymes between members of the *C. gattii* and *C. neoformans* species complexes. Unfortunately limited data on these enzymes have been published, and in that study no distinction was made between genotypes or species (Vidotto et al., 2006).

4.4. Genetic crosses

Detailed analyses of genetic crosses among and between isolates of species candidate groups, including the F1 and F2 generations are needed to observe reproductive barriers to approximate biological species concept. Unfortunately, most of the mating experiments done in the *C. gattii*/*C. neoformans* species complex do not fulfill these requirements. In many cases just a few tester strains, belonging to e.g., *C. deneoformans* (previously *C. neoformans* var. *neoformans*, AFLP2/VNIV, e.g., JEC20, JEC21) or *C. bacillisporus* (previously *C. gattii* AFLP5/VGIII, e.g., B4546, NIH312) have been used to induce a mating response. In almost all cases the observations stopped when hyphae and clamp connection and/or basidia were observed. Detailed genetic analyses of the F1 have usually not been made, and as far as we know none of the F2 generation. This hampers interpretation of results from mating experiments for taxonomic purposes. This is particularly true as many sexual reproduction strategies occur in the complex (see e.g., Nielsen and Heitman, 2007), including hybridization. The presence of hybrid isolates in the complex, such as the serotype AD, BD and AB hybrids (Aminnejad et al., 2012; Boekhout et al., 2001; Bovers et al., 2006, 2008b; Cogliati et al., 2006, 2011; Lengeler et al., 2001; Li et al., 2012a; Lin et al., 2007, 2008; Litvintseva et al., 2007; Hagen et al., 2010a, 2012b; Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009; Sun and Xu, 2007; Vogan and Xu,

2014; Xu et al., 2002; Xu and Mitchell, 2003) may, however, provide an argument that the parental isolates of these hybrids are genetically isolated and represent species. This is strongly supported by recent observations in which Bateson–Dobzjansky–Mul ler incompatibility affected the viability of basidiospores that resulted from crosses between isolates of serotype A and serotype D (Vogan and Xu, 2014). If compared to crosses among serotype A isolates and among serotype D isolates, the hybrid crosses yielded low numbers of fertile offspring suggesting that the putative parental groups are post-zygotically separated (Kwon-Chung and Varma, 2006; Lengeler et al., 2001; Lin et al., 2007), and are unable to go through normal meiosis. Kwon-Chung and Varma (2006) analyzed crosses between strains H99 (serotype A, genotype AFLP1/VNI) and JEC20 (serotype D, genotype AFLP2/VNIV) and between CBS6289 (serotype B, genotype AFLP4/VGI) and B3502 (serotype D, genotype AFLP2/VNIV). The 20 offspring isolates of the serotype AD cross were largely diploid or aneuploid, i.e., 70%, with complex serotypes, mating-types and mixed serotype AD, A or D allelic patterns of 5 genes. Three haploid isolates agreed with JEC20, and three were thought to be recombinants as they had the serotype A allele of *CNLAC* and serotype D alleles of *CAP59* and *URA5*. However, as the diploid or aneuploid genomes are highly dynamic one cannot rule out that the observed recombinants are not the result of a meiosis, but of post-zygotic chromosome/gene sorting. The same isolates were also crossed by Cogliati et al. (2006) with similar results, except that no haploid recombinants were observed. A crossing between the serotype A isolate CDC15 and serotype D isolate JEC20 showed a much shorter genome map length and a much higher physical distance to genetic distance ratios for this cross when compared to crosses among serotype A or among serotype D isolates (Sun and Xu, 2007). Lin et al. (2007) noted that serotype AD hybrids are inefficient in meiosis and sporulation and remained in the diploid state. Serotype AD diploid strains were found to be either sterile (70%) or self-fertile (30%) (Lengeler et al., 2001) and thus mating incompetent. Analysis of another serotype A × serotype D mating yielded a hybrid F1 with AD phenotype and genotype (Tanaka et al., 2003). These authors concluded that serotype AD isolates are diploid for many but not for all genomic loci. Another study demonstrated highly dynamic genomes with extensive loss of entire or partial chromosomes in serotype AD strains (Li et al., 2012a) that resulted in variable phenotypes, e.g., related to the serological epitopes. Serotype AD hybrid isolates have arisen by multiple recent (0-ca. 2 myr) hybridization events (Xu et al., 2002).

A crossing between strain CBS6289 (serotype B, genotype AFLP4/VGI) and B3502 (serotype D, genotype AFLP2/VNIV) yielded 50% diploids or aneuploids and 50% parental D strains. No haploid recombinants were recovered (Kwon-Chung and Varma, 2006). This, in our opinion, indicates that these two strains are genetically separated and the lack of recombinants suggests a considerable genetic distance. Part of the serotype BD diploids were phenotypically unstable in that the serotype BD phenotype switched to the serotype D phenotype (Kwon-Chung and Varma, 2006).

Within the *C. gattii* species complex crosses between strains of AFLP5/VGIII and AFLP6/VGII were performed and compared with crosses among AFLP6/VGII strains (Voelz et al., 2013). Intergenotypic crosses resulted in low rates of spore germination (<1%), low rates of successful meiosis with one crossing giving two out of 16 progeny being haploid recombinants and the other cross yielding diploid progeny only. Crosses among AFLP6/VGII isolates yielded a higher number of viable recombinant basidiospores (Voelz et al., 2013). Thus the results from the intergenotypic crosses largely corroborated that they represent genetically isolated cryptic species that are separated by post-zygotic isolation barriers. Kwon-Chung et al. (1982) studied a crossing between ATCC34874 (serotype D, genotype AFLP2/VNIV) and ATCC32609 (serotype C, genotype AFLP5/VGIII) and reported two types of basidiospores. Progeny of one basidium seemed to represent the serotype D parental with an 1:1 segregation of mating-types, whereas the progeny of the second basidium studied showed an 1:1 ration of Mal+ and Mal− strains. Another crossing presented by these authors was between ATCC32269 (serotype B, genotype AFLP4/VGI) and ATCC34874 (serotype D, genotype AFLP2/VNIV), viability of the basidiospores was 25–30% and 27% were self-fertile. A third crossing was between two other genotypic groups of the *C. gattii* species complex. Strain ATCC32269 (serotype B, genotype AFLP4/VGI) × strain ATCC32608 (serotype C, genotype AFLP5/VGIII) produced 30% fertile basidiospores and 30% of them were self-fertile. Phenotypic analysis of the offspring does not provide clues whether recombination has occurred. Unfortunately, the ploidy of the offspring of these last three crossings was not assessed and a detailed genetic analysis not performed. In our opinion, it cannot be ruled out that the basidia were the result of interspecies crossing with diploid or aneuploid basidiospores. Similarly, all but one of the crossings between ATCC36555 and ATCC28958 (serotype D, genotype AFLP2/VNIV) performed by Schmeding et al. (1981) represented interspecies crossings. Although the images of the basidia presented are somewhat difficult to interpret and a genetic analysis of the offspring was not made, their presence clearly suggest that post-zygotic isolation occurs between many genotypic groups, i.e., species.

Evidence of mitochondrial recombination was observed between various lineages of the *C. gattii* species complex, notably between genotype AFLP4/VGI and both genotype AFLP6/VGII and genotype AFLP7/VGIV (Bovers et al., 2009). All the observed genetic mechanisms acting within and between members of the *C. gattii*/*C. neoformans* species complex renders this a genetically highly dynamic evolutionary complex generating significant genotypic and phenotypic diversity that may provide fitness advantages for the various species and lineages under diverse environmental conditions. Interspecies hybrids are not limited to fungi, but occur in many lineages of the eukaryote tree of life, including fungi, plants and animals (Arnason et al., 1991; Benirschkek et al., 1964; Megersa et al., 2006; Mine et al., 2000; Weiss-Schneeweiss et al., 2013). In a very recent study mitochondrial inheritance was studied within and between isolates belong to various genotypes [read species] in the *C. gattii* species complex (Wang et al., 2015). Interestingly, various inheritance patterns as well as recombinants were observed. Recombinants were seen in three out of seven intra molecular type VGIII crosses, in two of the molecular type VGII × VGII crosses and in 3 out of molecular type VGI × VGIII crosses, thus corroborating previous observations on the presence of mitochondrial recombination in the *C. gattii* species complex (Bovers et al., 2009). As the main mechanism of speciation in the *C. gattii*/*C. neoformans* species complex is regulated by post zygotic isolation mechanisms [see above] it remains to be seen how these patterns of mitochondrial recombination play a role in the speciation processes.

With the data at hand, however, we demonstrate evidence from different analyses that the *C. gattii*/*C. neoformans* species complex consists of more species than currently accepted, in agreement with other groups of fungi (Crespo and Lumbsch, 2010; Hibbett and Taylor, 2013: Lumbsch and Leavitt, 2011; Taylor et al., 2000). The seven-species concept for the *C. gattii*/*C. neoformans* species complex, adopted here, represents the minimal species diversity in the group and additional data will be necessary to elucidate the taxonomic status of clades F–H. This seven species concept is supported by the phylogenetic species concept and largely corroborated by available phenotypic data and information on post-zygotic isolation barriers that can be interpreted as a proxy for the biological species

concept. However, we cannot rule out that further genetic and phylogenetic studies will unveil other cryptic species in the complex, e.g., in *C. neoformans*.

4.5. Virulence and susceptibility to antifungals

Only few studies have addressed comparative aspects of virulence among all members of the *C. gattii*/*C. neoformans* species complex. One study used *Galleria mellonella* as a model host to compare the molecular types of *C. gattii* (Firacative et al., 2014). No relationships were found between the molecular types and cell size, capsule thickness, production of melanin, and growth rate at 37 °C. Although not statistically significant, the authors noted that isolates of genotype AFLP4/VGI (=*C. gattii sensu stricto* in new taxonomy) caused a higher proportion of death in a shorter time than strains of genotypes AFLP5/VGIII (=*C. bacillisporus* in new taxonomy), AFLP6/VGII (=*C. deuterogattii* in new taxonomy), and VGIV (either *C. tetragattii* or *C. decagattii* in new taxonomy). Another study used *Drosophila* as a model host (Thompson III et al., 2014). These authors investigated most genotypic groups in the complex [note that they did not include genotype AFLP2/VNIV (=*C. deneoformans* in new taxonomy) nor the serotype AD hybrids] and found that genotype AFLP5/VGIII (=*C. bacillisporus* in new taxonomy) was the most virulent group. Growth at 30 °C was also highest for isolates of this group, but at 37 °C this was genotype AFLP1/VNI (=*C. neoformans* in new taxonomy). Similar to our results capsules were thickest for *C. gattii* isolates, especially genotypes AFLP4/VGI (like our results) and AFLP5/VGIII (unlike our data). These authors also showed that at 30 °C, genotype AFLP1/VNI isolates produced more melanin than the *C. gattii* species complex genotypes. Similarly isolates of genotype AFLP1/VNI showed better tolerance to hydrogen peroxide. They also noted differences in susceptibility to antifungals between the so-called genotypic groups (Thompson III et al., 2009, 2014). MICs to fluconazole and itraconazole were higher in genotype AFLP6C/VGIIc (=*C. deuterogattii* subtype C in new taxonomy) than in genotypes AFLP4/VGI (=*C. gattii* in new taxonomy) and AFLP6/VGII (=*C. deuterogattii* in new taxonomy), as well as genotype AFLP1/VNI (=*C. neoformans* in new taxonomy) for fluconazole. MICs for posaconazole were higher for genotype AFLP6C/VGIIc than for genotype AFLP1/VNI. According to these authors survival of *Drosophila* was related to molecular type (=species) or subtype (=intraspecies). Virulence in immunocompetent mice of a strain of *C. gattii* (genotype AFLP4/VGI) that was isolated from a goat in Spain was compared with that of a *C. deneoformans* (serotype D) and a *C. bacillisporus* (serotype C) isolate (Torres-Rodríguez et al., 2003). In all cases mortality was low (2%), but the highest rates of positive cultures from tissue were from *C. gattii*, followed by *C. deneoformans* and *C. bacillisporus*. Finally, it was observed that *C. neoformans* (genotype AFLP1/VNI) grew faster in the brain and *C. deuterogattii* (cited as *C. gattii* genotype AFLP6/VGII) in the lungs of C57BL/6 and BALB/c mice (Ngamskulrungroj et al., 2012b). Moreover, growth in blood by *C. deuterogattii* was slower than that of *C. neoformans*. In brief, these and our studies suggest differences in virulence and pathogenicity of, at least, part of the new species in the *C. gattii*/*C. neoformans* species complex. It is, however, likely that significant strain-based differences exist in virulence within the species as was demonstrated using tail-vein inoculation experiments in mice and macrophages using many globally collected isolates of *C. deuterogattii* (Hagen et al., 2013).

The first practice guidelines for the treatment of cryptococcal infections were established by Saag et al. (2000) who described several treatment strategies for different patient groups. These include monotherapy with fluconazole for immunocompetent individuals and combined amphotericin B and 5-fluorocytosine induction therapy followed by a prolonged fluconazole consolidation therapy (Saag et al., 2000). The outcomes of two large epidemiological studies initiated by the French Cryptococcosis Study Group pleaded for updated guidelines (Dromer et al., 2007, 2008). A decade after the first consensus practice guidelines for the treatment of cryptococcosis, a thorough update was provided by Perfect et al. (2010). These updated practice guidelines include an induction therapy with amphotericin B and 5-fluorocytosine with different doses for specific groups (e.g., patient groups, clinical presentation, *C. gattii* versus *C. neoformans* species complexes), followed by a period of consolidation with fluconazole. Resistance has been reported for these antifungals, especially for fluconazole when used as monotherapy (Pan et al., 2012; Sar et al., 2004; Van Wyk et al., 2014). A review on cases of fluconazole resistant clinical isolates showed that this might be due to monotherapy but that heteroresistance can be a potential alternative route (Cheong and McCormack, 2013; Sionov et al., 2009). Hence the necessity to perform routine testing of MICs for clinical cryptococcal isolates remains.

4.6. Identification of species

Rapid and reliable identification of clinically important fungi is highly important. Fortunately, a number of recently developed approaches make this feasible for the species of the *C. gattii*/*C. neoformans* species complex. One such technique is MALDI-TOF MS that has been successfully applied to identify and separate closely related pathogenic yeasts (Cendejas-Bueno et al., 2012; Firacative et al., 2012; Kolecka et al., 2013). Correct identification of *C. gattii* and *C. neoformans* and their genotypes by MALDI-TOF MS was reported (Firacative et al., 2012; Hagen et al., 2011; McTaggart et al., 2011; Posteraro et al., 2012). These results are largely confirmed by our results for which a test set of 425 well characterized isolates was used. Major errors, i.e., as incorrect genus identification, were not observed and minor errors, viz. misidentification at the species level, were found occasionally between hybrid strains. Use of media known to impact capsule development, i.e., SGA versus SGA + 0.5M NaCl, did not influence the identification results. Thus, MALDI-TOF MS is an excellent method to identify members of the *C. gattii*/*C. neoformans* species complex reliably at the species level. PCR-RFLP analysis of the *CAP1* and *GEF1* genes also allowed identification of all species and their mating-types in the *C. gattii*/*C. neoformans* species complex (Feng et al., 2008a). Hyperbranched rolling circle amplification (HRCA) using species-specific (referred to as molecular types by the authors) padlock probes based on the *PLB1* gene allowed discrimination of all species (Trilles et al., 2014). Other studies separated *C. neoformans*, *C. deneoformans* and the *C. gattii* species complex (Feng et al., 2013; Kaocharoen et al., 2008) by singleplex PCR analysis of the *STR1* gene and a HRCA targeting the ITS, respectively. Older studies using the Luminex *x*MAP technology with genotype and species specific primers based on the IGS1 region were able to identify all the newly recognized species, but we have to keep in mind that isolates of *C. tetragattii* and *C. decagattii* were not available at that time (Bovers et al., 2007; Diaz and Fell, 2005b) and need further testing. To some extent this method successfully identified *Cryptococcus* cells directly from infected CSF (Bovers et al., 2007).

4.7. The position of CBS132 as type strain and naming the species

After we recognize the species, a further point of concern is how to name them? Strain CBS132 is assumed to represent an original isolate made by Sanfelice (1895) and was selected as the type strain of *C. neoformans* (dried material in Herbarium BPI802707, ex-type culture CBS132) which is the conserved type species of the genus *Cryptococcus* (Fonseca et al., 2011; Kwon-Chung, 2011; McNeil et al., 2006). This strain was send to CBS-KNAW by

Giordano, who stated in his 1939 study that he possessed the original isolate made by Sanfelice. However, in the 3rd edition of the reference work 'The Yeasts – A taxonomic study' (Phaff and Fell, 1970) the authors were not fully convinced that this strain originated from Sanfelice as they wrote 'One strain, received by the CBS in 1935 from Giordano in Italy, *was assumed* [italics by present authors] by Lodder and Kreger-van Rij (1952) to be the original strain of Sanfelice and was chosen by them as the type strain'. Various attempts to assess the serotype of isolate CBS132 yielded different results. Serological studies, including a dot enzyme assay, multi-locus enzyme typing, AFLP analysis, and PCR and dot blot analysis of mating-type pheromones showed it to be a serotype AD hybrid (Belay et al., 1996; Boekhout et al., 2001; Brandt et al., 1993; Cogliati et al., 2001; Ikeda et al., 1996, 2000; Kabasawa et al., 1991). Kwon-Chung et al. (1978) reported that it had serotype D as did Dromer et al. (1993) who found an immunofluorescence pattern consistent with serotype D by using a monoclonal antibody against capsular polysaccharides. The latter authors noted the discrepancy with the reported serotype AD status. In contrast, Lengeler et al. (2001) reported serotype A for this strain using the CryptoCheck serotyping kit from Iatron Laboratories. The same authors found that by using PCR analysis several mating-type locus linked genes gave either a serotype A + D, an A, or a D-specific pattern, flow cytometry yielded a diploid genome size, and the isolate was found to be sterile. Using a set of *CAP59* primers, Lucas et al. (2010) only found a serotype D allele and suggested that the serotype A allele was lost. Since CBS132 was selected as holotype of *C. neoformans* variety *neoformans* (=serotype D) after recognition of *C. neoformans* variety *grubii* (=serotype A) with type strain H99 (=CBS8710 = CBS10515) by Franzot et al. (1999), the proper genetic characterization of this strain became even more important. These authors, however, also stated that serotype may not be a stable phenotypic character. Taken together these data strongly suggest that CBS132, which most likely represent an authentic isolate made by Sanfelice, is a serotype AD hybrid isolate. This, in our opinion, disqualifies this strain as the type strain of *C. neoformans* as it has genetic and phenotypic properties of both serotype A and serotype D background. The hybrid nature of CBS132 also makes it difficult, if not impossible, to decide whether *C. neoformans* as described by Sanfelice (1895) represents serotype D [as it is seen by most contemporary researchers] or serotype A? In our opinion it is best to interpret it simply as a serotype AD hybrid that originated as a result from a cross between *C. neoformans* representing serotype A, and '*C. deneoformans*' representing serotype D (see below for the new taxonomy).

Serotype A isolates are by far clinically more relevant, both in number of patients and the typical meningitis it usually causes (Park et al., 2009). Thus cryptococcal meningitis is mainly caused by *C. neoformans* serotype A isolates, especially in HIV-infected patients, and globally causes an estimated one million new infections with over 625,000 casualties each year (Park et al., 2009). Thus at a global scale cryptococcosis is clearly connected to serotype A isolates, which, in our opinion, favors the naming of the species representing serotype A isolates as *C. neoformans*. We realize that this opposes the proposal made by Franzot et al. (1999) in which varietal status was given to serotype A isolates as *C. neoformans* variety *grubii* Franzot, Salkin & Casadevall. However, we assume that the clinical field will benefit most from naming serotype A strains as *C. neoformans* as this clearly links the most common pathogen with the major disease it causes.

For selecting a new type strain of *C. neoformans* two options seem preferable. The first option is to select the putative original isolate of *Saccharomyces hominis*, CBS879 (=ATCC4189) made by Busse (1895) that is serotype A (Drouhet, 1997) and genotype AFLP1/VNI (Boekhout et al., 2001). Moreover, based on the sequence analysis presented here it belongs to the core cluster of *C. neoformans* serotype A. Unfortunately, this strain has not been studied widely. The second option is to select a strain that has been widely studied. In this aspect we agree with Franzot et al. (1999) who selected strain H99 (=CBS8710 = CBS10515) as holotype for variety *grubii* (=serotype A isolates), because the strain is widely used as an experimental model in many studies, and its genome has been released recently (Janbon et al., 2014). Belay et al. (1996) confirmed the serotype A status of the isolate. We favor this last option to keep H99 as epitype for *C. neoformans*.

If we discard CBS132 as a type strain, this also implicates that a new type strain has to be selected to represent the species agreeing with serotype D for which we propose the name *C. deneoformans* to remind the close relationship with *C. neoformans* (See Taxonomy below). Similarly as for H99, we consider that strain B3501 (=CBS6900) is a good representative for this genotypically and serologically well characterized species. This isolate is genetically well studied, it has been widely used as a model strain in many experiments, it is serotype D (Belay et al., 1996) and the analysis of its genome has set the standard of the field (Loftus et al., 2005). Thus we propose to use CBS6900 as holotype for *C. deneoformans* (see Taxonomy below] with ex-type strains CBS6900 = B3501.

In 2002, *C. gattii* (Vanbreuseghem & Takashio) Kwon-Chung & Boekhout was proposed as a new species and conserved against *C. bacillisporus* Kwon-Chung & J.E. Bennett and *C. hondurianus* Castellani (Kwon-Chung et al., 2002). More than a century ago, Curtis (1896) described *Saccharomyces subcutaneous tumefaciens* that turned out to represent *C. gattii* (Drouhet, 1997). Serologically, the original Curtis' isolate CBS1622 (=ATCC2344) belongs to serotype B (Drouhet, 1997) and was found to belong to genotype AFLP4/VGI (Boekhout et al., 2001; Hagen et al., 2012b). Vanbreuseghem and Takashio (1970) described *C. neoformans* var. *gattii* based on a specimen that was isolated from spinal fluid of a man in Kinshasa, Congo, now Democratic Republic of Congo (Africa). In 1978 *C. bacillisporus* was described (Kwon-Chung et al., 1978) and, consequently, when both species are considered to be conspecific only one valid name could be used. Since the involvement of *C. gattii* in a number of globally emerging outbreaks this name has largely gained popularity and this was one of the arguments to conserve the name *C. gattii* (Vanbreus. & Takashio) Kwon-Chung & Boekhout over *C. bacillisporus* Kwon-Chung & J.E. Bennett (Kwon-Chung et al., 2002). Presently, we propose to describe all five major genotypic lineages present in the *C. gattii* complex (Figs. 1 and 2) as species. The type strains of *C. gattii* RV20186 (=CBS6289 = ATCC32269 = MUCL30449) belongs to genotypic group AFLP4/VGI, and has serotype B (Belay et al., 1996; Boekhout et al., 1997, 2001), whereas that of *C. bacillisporus* ATCC32608
(=CBS6955 = CBS6916 = DBVPG6225 = MUCL30454 = NIH191) affiliates with genotypic group AFLP5/VGIII and has serotype C (Boekhout et al., 1997, 2001). Thus, these two type strains represent different species (see below), despite that they were mating competent. Thus the name *C. bacillisporus* has to be used for the AFLP5/VGIII genotypic cluster. For the other three genotypic lineages in the *C. gattii* species complex, namely the genotypic clusters AFLP6/VGII, AFLP7/VGIV and AFLP10/VGIV, no older names are available and new names will be proposed that retain '*gattii*' in order to link them to the *C. gattii* species complex (see below Taxonomy).

4.8. One fungus = One name nomenclature

In the 5th edition of the reference work 'The Yeasts – A taxonomic study' (Kurtzman et al., 2011a) both *C. neoformans* and *C. gattii* are treated under the teleomorphic genus *Filobasidiella* (Kwon-Chung, 1976, 2011; Kwon-Chung et al., 1982). With the current 'One fungus = One name' concept for naming fungal species (Hawksworth et al., 2011) using two names for the same

species is no longer accepted. This renders the question whether to use the asexual (i.e., *Cryptococcus*) or the sexual (i.e., *Filobasidiella*) name? As the name *Cryptococcus* is also the denominator for the disease cryptococcosis, and because it is much more widely used we favor to use *Cryptococcus* above *Filobasidiella*. For instance, in Google Scholar searching *Filobasidiella* resulted in 2680 hits, *Cryptococcus* gave 115,000 hits and cryptococcosis 29,100 hits (February 10th, 2015). The '*Filobasidiella*' clade includes also *F. lutea* and *F. depauperata* (Kwon-Chung, 2011) and these two species have to be renamed under *Cryptococcus* if that genus name is to be used for the clade. A further complication is that the genus *Cryptococcus* as it is currently recognized is highly polyphyletic with species distributed in all four orders of Tremellomycetes (Fonseca et al., 2011; Millanes et al., 2011). The genus is in urgent need for revision using monophyly as discerned by multigene or comparative genomics analyses as the leading principle. Thus, if the *Filobasidiella* lineage is considered to represent the genus *Cryptococcus*, by far the majority of currently named cryptococcal species need to be renamed. A phylogenetic analysis by using six genes of most currently described yeast and yeast-like species in Tremellomycetes is underway and this will provide a base for reclassification and renaming the currently named *Cryptococcus* species in Tremellomycetes (Liu et al., 2015).

5. Taxonomy

5.1. Cryptococcus neoformans (Sanfelice) Vuillemin

In: Vuillemin, P., 1901, Les blastomycètes pathogens. Rev. Gen. Sci. 12: 732–751.

Serotype A (Belay et al., 1996; Boekhout et al., 1997, 2001), genotype AFLP1, AFLP1A, AFLP1B (Boekhout et al., 2001; Bovers et al., 2008a), AFLP genotype VNB (Litvintseva et al., 2006), PCR fingerprint and *URA5*-RFLP molecular type VNI and VNII (Meyer et al., 2009, 2011).

Mycobank: MB119294.

Epitype NYSD 1649, New York State Herbarium, Albany, New York, U.S.A. Herbarium. Holotype of *C. neoformans* var. *grubii*. Ex-type material also preserved as CBS H-21964. Ex-type cultures CBS8710 = H99 = CBS10515.

Isolated by A. Proctor, Durham, North Carolina, U.S.A. from patient with Hodgkins lymphoma.

Genotype AFLP1, serotype A, mating-type α (Bovers et al., 2008a). Isolated from a patient with Hodgkins lymphoma, A. Proctor > J. Perfect, Durham, North Carolina, U.S.A.

5.1.1. Confirmed synonyms

Saccharomyces neoformans Sanfelice, 1894. In: Sanfelice, F., 1894, Contributo alla morfologia e biologia dei blastomiceti che si sviluppano nei succhidi alcuni frutti. Ann. Igien. 4: 463–495. Authentic strain CBS132 [=serotype AD hybrid].

Debaryomyces neoformans (Sanfelice) Redaelli, Ciferri & Giordano, 1937. In: Redaelli, P., Ciferri, R., Giordano, A., 1937, *Debaryomyces neoformans* (Sanfelice) nobis n. comb. pour les espèces du groupe *Saccharomyces hominis-Cryptococcus neoformans-Torula histolytica*. Boll. Sez. Ital. Soc. Int. Microbiol. 9: 24–28.

Cryptococcus hominis Vuillemin, 1901. In: Vuillemin, P., 1901, Les blastomycètes pathogens. Rev. Gen. Sci. 12: 732–751. CBS879 = ATCC4189 is a possible authentic isolate of the strain that was isolated by Busse (1895) and named *C. hominis* by Vuillemin (1901). According to Lodder & Kreger-van Rij (1952) strains from this species were received in 1924 from Ota in Paris and from Voss in 1925, which makes the identity of CBS879 questionable with respect to its authenticity.

Saccharomyces hominis Constantin, 1901. Cited as a synonym under *Filobasidiella neoformans* by Kwon-Chung (2011). As this most likely also represent the species found by Busse the synonymy under *C. neoformans* may be correct.

Atelosaccharomyces hominis (Vuillemin) Todd & Herrman, 1936. In: Todd, R.L., Herrmann, W.W., 1936, The life cycle of the organism causing yeast meningitis. J. Bact. 32: 89–97.

Torulopsis hominis (Vuillemin) Redaelli, 1931. In: Redaelli, P., 1931, Il problema della Torulopsidaceaea e dei loro rapporti con lúomo e con la patologia umana studiato particularmente in Italia. (Blastomiceti e blstimicosi). Riv. Biol. 13: 171–235.

Candida psicrophylicus Niño, 1934. In: Speroni D, Llambías J, Parodi SE, Niño FL. 1934, Blastomicosis humana generalizada por criptococo (n. sp.). Estudio parasitológico, anátomopatoló gico, clínico y experimental. Bol. B. Aires Univ. Nac. Inst. Clin. Quir. 5:94–155. Authentic strain CBS996, genotype AFLP1, serotype A (Boekhout et al., 2001).

Cryptococcus neoformans (Sanfelice) Vuillemin variety *grubii* Franzot, Salkin & Casadevall. In: Franzot et al., 1999, *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J. Clin. Microbiol. 37: 839. Holotype NYSD1649, New York State Herbarium, Albany, New York, U.S.A. Ex-type cultures H99 = CBS8710 = CBS10515, genotype AFLP1, serotype A (Boekhout et al., 2001). Note that in Franzot et al. (1999) *Filobasidiella neoformans* variety *neoformans* is listed as the teleomorph for *C. neoformans* variety *grubii*. The holotype of this species, BPI71853 is, however, based on a crossing between NIH12 (=CBS6885) × NIH430 (=CBS6886) (Kwon-Chung, 1975), which both are serotype D strains (Boekhout et al., 1997, 2001; Martinez et al., 2001). This incongruence between the anamorphic and teleomorphic species concepts with respect to serotype designation makes this anamorph-teleomorph connection incorrect.

Note: In The Yeasts, a taxonomic study, 5th edition, many synonyms are listed for *Cryptococcus neoformans*, the anamorph of *Filobasidiella neoformans* (Kwon-Chung, 2011). For most of them no material is available for study and thus the actual species designation could not be confirmed. Consequently, these names have to be rejected.

Description based on strains CBS996, CBS8710, CBS8336, CBS10085 (=WM148), CBS10515, P152.

After 3 days at 25 °C in 3% glucose medium, sediment is present; yeast cells are globose, usually with a single bud, but occasionally some cells may adhere, 4.0–7.0 × 4.0–7.0 μm. Bigger cells measuring up to 9 μm diameter are present, On YMoA, streak colonies are 5–6.5 mm width, smooth, moist to mucoid, shiny, creamish white, with an entire margin; cells are subglobose to globose, 4.0–6.0 μm in diameter, usually with one bud, but occasionally with two buds. On Dalmau plates only yeast cells are present. On MEA colonies measure 8–11 mm, and are pale yellowish beige (pale isabella), and becoming highly mucoid. The *Filobasidiella* teleomorph has dikaryotic hyphae, clamp connections, and cylindrical clavate basidia with four chains of somewhat elongate, slightly rough basidiospores (Litvintseva et al., 2003; Nielsen et al., 2003, 2007; Velagapudi et al., 2009). Serotype A (Boekhout et al., 1997, 2001; Dromer et al., 1993), genotype AFLP1 (Boekhout et al., 2001), PCR fingerprint and *URA5*-RFLP molecular type VNI (Meyer et al., 2009, 2011). Clades F–H in Fig. 1.

5.1.2. Occurrence and habitats

Cryptococcus neoformans was found to be the most commonly occurring species comprising 63% [genotypes AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII combined] of 2755 globally collected isolates (Meyer et al., 2011). Among the clinically and

veterinary isolates ($n = 1250$) this was even higher with 69% and among the environmental isolates somewhat lower with 42% (Meyer et al., 2011). Over 52% of the isolates came from HIV-infected patients and more than 16% from apparently immunocompetent people (Meyer et al., 2011). *C. neoformans* has a global occurrence (Mitchell et al., 2011) and is known from Europe (e.g., Austria, Belgium, Bulgaria, Croatia, France, Germany, Greece, Hungary, Italy, Netherlands, Poland, Portugal, Serbia, Spain, Switzerland, UK, Russia); Asia (e.g., China, India, Indonesia, Israel, Japan, Kuwait, Papua New Guinea, Philippines, Qatar, Thailand); Oceania (e.g., Australia, New Zealand); Africa (e.g., Botswana, Congo, Rwanda, South Africa, Zimbabwe); North America (Canada, U.S.A.); Central and South America (e.g., Argentina, Brazil, Colombia, Chile, Cuba, French Guiana, Guatemala, Mexico, Peru, Venezuela) (Arsic Arsenijevic et al., 2014; Cattana et al., 2013; Debourgogne et al., 2011; Escandón et al., 2006; Guinea et al., 2010; Hagen et al., 2012a; Illnait-Zaragozi et al., 2010; Khayhan et al., 2013; Meyer et al., 2011; Mlinaric-Missoni et al., 2011; Pan et al., 2012; Viviani et al., 2006). The percentage among environmentally obtained isolates ranged from 14% (Oceania), 30% (North America), 40% (Europe) to 47% (South America) (Meyer et al., 2011).

The species is known from pigeons, other birds, trees and soils (Mitchell et al., 2011). *C. neoformans* is widely isolated from droppings from a variety of birds, especially the common pigeon (*Columba livia*), but also from doves (e.g., spotted dove, *Melopsittacus unolatus*; zebra dove, *Geopelia striata*), ducks (white-faced whistling duck, *Dendrocygna viduata*), Indian peafowl (*Pavo cristatus*), cockatoos and other psittacine birds (e.g., *Cacatua roseicapilla*) (Filiú et al., 2002), passerine birds (e.g., canary, *Serinus canaria domestica*), birds of prey (e.g., spotted eagle-owl, *Bubo africanus*; buzzard, *Buteo buteo*; common kestrel, *Falco tinnunculus*; black kite, *Milvus migrans*), bird cages at a commercial aviary (Cafarchia et al., 2006; Costa Sdo et al., 2009). The species is reported to cause minimally subcutaneaous disease in pigeons (Malik et al., 2003). *Cryptococcus neoformans* is also reported from various mammals, such as dogs, cats, cows, bandicoot rats, striped grass mouse, gorilla, as well as various veterinary samples (eye, nose, blood, lung, kidney, ulcerated cheek, osteolytis, parasinal) and a hippopotamous cage (Bauwens et al., 2004; Boekhout et al., 2001; Danesi et al., 2014; Lugarini et al., 2008; Magalhães et al., 2012; Malik et al., 2003; Morera et al., 2014; O'Brien et al., 2004; Singer et al., 2014; Singh et al., 2007). Tree species from which *C. neoformans* has been isolated belong to the following families: Anacardiaceae (*Mangifera indica*), Annonaceae (*Polyaltha longifolia*), Apocynaceae (*Alstonia scholaris*), Bignoniaceae (*Tabebuia guayacan*), Chrysobalanaceae (*Licania* (*Moquilea*) *tomentosa*), Combretaceae (*Terminalia catappa*, *T. arjuna*), Cupressaceae (*Cupressus sempervirens*), Fabaceae (*Acacia decurrens*, *A. nilotica*, *Anadenanthera peregrina*, *Butea monosperma*, *Caesalpinia peltophoroides*, *Cassia fistula*, *C. grande*, *Colophospermum mopane*, *Dalbergia sissoo*, *Myroxylon peruiferum*, *Prosopis juliflora*, *Senna multijuga*, *S. siamea*, *Tamarindus indica*, *Tipuana tipu*), Fagaceae (*Quercus macrantha*), Lauraceae (*Laurus* species), Malvaceae (*Adansonia digitata*, *Theobroma cacao*), Meliaceae (*Azadirachta indica*), Moraceae (*Ficus microcarpa*, *F. religiosa*, *F. tequindamae*), Myrtaceae (*Eucalyptus camaldulensis*, *E. deglupta*, *E. globosus*, *Syzygium cucumi*), Palmae (*Phoenix* species), Pinaceae (*Cedrus deodara*, *Pinus* species), Rosaceae (*Prunus dulci*), Rubiaceae (*Guettarda acreana*), Rutaceae (*Aegle marmelos*), Sapotaceae (*Manilkara hexandra*, *Mimusops elengi*). Note that Fabaceae are represented with 15 species. Besides the species is known from a cockroach, soil, desert soil, house dust, decaying wood, debris, and pine needles (Boekhout et al., 2001; Chowdhary et al., 2011, 2012b; Colom et al., 2012; Costa Sdo et al., 2009; Grover et al., 2007; Illnait-Zaragozí et al., 2012; Mitchell et al., 2011; Mischnik et al., 2014; Randhawa et al., 2006; Refojo et al., 2009). The species is able to mate at woody debris of *Acacia mearnsii* and *Eucalyptus camaldulensis* (Botes et al., 2009).

5.1.3. Epidemiology

Cryptococcus neoformans has a worldwide occurrence and the majority of clinical isolates, predominantly isolated from HIV-infected or otherwise immunosuppressed individuals, belong to this species (Arsic Arsenijevic et al., 2014; Bii et al., 2007; Calvo et al., 2001; Chan et al., 2014; Cogliati, 2013; Dromer et al., 1994; Favalessa et al., 2014a; Govender et al., 2011; Guinea et al., 2010; Hagen et al., 2012a; Illnait-Zaragozí et al., 2010; Li et al., 2012b; Liaw et al., 2010; Pan et al., 2012; Pfaller et al., 1998; Poonwan et al., 1997; Sanchini et al., 2014; Souza et al., 2010; Thompson III et al., 2009; Tsujisaki et al., 2013; Viviani et al., 2006). Numerous globally initiated epidemiological studies reported that, irrespective of the source of the included *C. neoformans* isolates, they exclusively were of the mating-type α (Arsic Arsenijevic et al., 2014; Bii et al., 2007; Casali et al., 2003; Chen et al., 2008; Chowdhary et al., 2011; Escandón et al., 2006; Hagen et al., 2012a; Hiremath et al., 2008; Feng et al., 2008b; Guinea et al., 2010; Litvintseva et al., 2005; Lugarini et al., 2008; Matsumoto et al., 2007; Mihara et al., 2013; Park et al., 2014b; Romeo et al., 2012; Sanchini et al., 2014; Simwami et al., 2011; Yan et al., 2002), with the exception of a few that reported mating-type **a** isolates (Freire et al., 2012; Hsu et al., 1994; Litvintseva et al., 2006; Viviani et al., 2006) (Supplementary Table 4).

There are indications that infections with *C. neoformans* are due to a genetic risk profile among HIV-infected individuals (Netea, 2013; Rohatgi et al., 2013) and that an altered immune response enhance *C. neoformans* infection in mice (Leongson et al., 2013). However, in South-East Asian cryptococcosis patients the majority of isolates were observed to be HIV-negative (Chau et al., 2010; Chen et al., 2008; Choi et al., 2010; Pan et al., 2012), but also at other localities *C. neoformans* infections among immunocompetent individuals have been found (Hagen et al., 2012a; Liaw et al., 2010; Mlinaric-Missoni et al., 2011). It has been observed that inhibitory Fc receptors, FcγRIIB, may play a role in cryptococcal infection in such apparently immunocompetent individuals (Hu et al., 2012).

5.1.4. Susceptibility to antifungals

Isolates are usually susceptible to the standard clinically used antifungals, including amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole, voriconazole, and the novel antifungal isavuconazole (Arsic Arsenijevic et al., 2014; Bii et al., 2007; Calvo et al., 2001; Chowdhary et al., 2011; de Fernandes et al., 2003; Guinea et al., 2010; Hagen et al., 2012a; Illnait-Zaragozi et al., 2010; Kobayashi et al., 2005; Lee et al., 2012; Li et al., 2012b; Liaw et al., 2010; Mlinaric-Missoni et al., 2011; Morera-López et al., 2005; Pan et al., 2012; Pfaller et al., 1998; Poonwan et al., 1997; Souza et al., 2010; Tewari et al., 2012; Tsujisaki et al., 2013). Some isolates showed elevated MICs or resistance to amphotericin B (Andrade-Silva et al., 2013; Lee et al., 2012; Li et al., 2012b; Matos et al., 2012), 5-fluorocytosine (Arsic Arsenijevic et al., 2014; Bii et al., 2007; Calvo et al., 2001; Chowdhary et al., 2011; Hagen et al., 2012a; Matos et al., 2012; Pan et al., 2012), fluconazole (Andrade-Silva et al., 2013; de Fernandes et al., 2003; Hagen et al., 2012a; Lee et al., 2012; Matos et al., 2012; Morera-López et al., 2005; Pan et al., 2012; Sar et al., 2004; Tangwattanachuleeporn et al., 2013; Tewari et al., 2012) and itraconazole (Bii et al., 2007). Resistance to fluconazole has been described in 20 cases, but therapeutic failure cannot be fully attributed to altered susceptibility (Cheong and McCormack, 2013). Fluconazole resistant isolates were also observed in India, Indonesia and Thailand as were five fluconazole

and 5-fluorocytosine double resistant isolates in Indonesia (Khayhan et al., 2013; Pan et al., 2012). Isolates of *C. neoformans* genotype AFLP1/VNI were found to have a significantly higher geometric mean MIC for fluconazole than isolates of the two subgenotypes AFLP1A/VNB/VNII and AFLP1B/VNII (Chong et al., 2010). *C. neoformans* has been observed to be more susceptible to several antifungals when compared to *C. gattii* (genotype AFLP4/VGI) (Chowdhary et al., 2011) and *C. deuterogattii* (genotype AFLP6/VGII) (Trilles et al., 2012), but less susceptible for 5-fluorocytosine and amphotericin B when compared to *C. gattii* (Chau et al., 2010).

Epidemiological cutoff values (ECVs) were recently established, with ECVs for amphotericin B 0.5 μg/ml for genotype AFLP1/VNI and 1 μg/ml for non-genotyped *C. neoformans* isolates; 5-fluorocytosine 8 μg/ml and 16 μg/ml for non-typed *C. neoformans* isolates; fluconazole 8 μg/ml and 16 μg/ml for non-genotyped *C. neoformans* isolates; itraconazole 0.25 μg/ml and 0.5 μg/ml for non-genotyped *C. neoformans* isolates; posaconazole and voriconazole 0.25 μg/ml *C. neoformans* and non-genotyped *C. neoformans* isolates; and isavuconazole 0.06 μg/ml for non-genotyped *C. neoformans* isolates and 0.12 μg/ml for genotype AFLP1/VNI isolates (Espinel-Ingroff et al., 2012a,b, 2015).

5.1.5. Virulence

Isolates of *C. neoformans* showed the highest growth rate at 37 °C compared to the other species (referred to as genotypes, Thompson III et al., 2014). A difference in fungal burden has been observed between male and female mice, with the former being more prone to have a higher fungal burden (Carroll et al., 2008). Furthermore, pathogenicity assays showed that macrophages from human females phagocytise more *C. neoformans* cells than those obtained from human males, but that those from the latter were found to be faster killed by cryptococcal cells (McClelland et al., 2013). On the contrary, by using the nematode *Caenorhabditis elegans* it was observed that male nematodes were more resistant against *C. neoformans* infection compared to hermaphrodite nematodes (Van den Berg et al., 2006). Virulence of *C. neoformans* (cited as molecular type VNI, Thompson III et al., 2014) as assessed in a *Drosophila* host was lower if compared to *C. bacillisporus* (cited as molecular type VGIII). Similarly, growth rates at 30 °C were also lower of compared to *C. bacillisporus*, but at 37 °C growth rates of *C. neoformans* (cited as molecular type VNI) were highest. Similarly at 30 °C on L-DOPA medium the species produced more melanin than *C. gattii* (cited as molecular type VGI), *C. bacillisporus* (cited as molecular type VGIII), C. *deuterogattii* (cited as molecular type VGII) and *C. tetragattii* (cited as molecular type VGIV). Capsule size was less than that of all members of the *C. gattii* species complex (Thompson III et al., 2014).

5.2. Cryptococcus deneoformans Hagen & Boekhout, sp. nov.

Etymology: 'de'neoformans, indicating that it represents the serotype D ['de'] isolates of the *C. neoformans* species complex, as well as to indicate that the species is not the proper *C. neoformans*, with 'de' meaning 'off'.

Mycobank: MB810281.

Holotype CBS H-21965. Ex-type cultures CBS6900 = CBS7697 = B-3501 = DBVPG6228. Isolated by K.J. Kwon-Chung, Bethesda, Maryland, U.S.A., from crossing.

Genotype AFLP2, serotype D, mating-type α (Boekhout et al., 2001; Bovers et al., 2008a).

5.2.1. Confirmed synonyms

Saccharomyces neoformans Sanfelice, 1894. In: Sanfelice, F., 1894, Contributo alla morfologia e biologia dei blastomiceti che si sviluppano nei succhidi alcuni frutti. Ann. Igien. 4: 463–495. Type CBS132 [=serotype AD hybrid].

Filobasidiella neoformans Kwon-Chung (1975). In: Kwon-Chung, K.J., 1975, A new genus *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia 67: 1197–1200. This teleomorph description was based on crossing NIH12 (=CBS6885) × NIH430 (=CBS6886) (Kwon-Chung, 1975), which both are serotype D and belong to genotype AFLP2/VNIV (Boekhout et al., 1997, 2001; Bovers et al., 2008a; Martinez et al., 2001). The holotype BPI71843, a result from the above indicated crossing, is deposited in the National Fungus Collection, Beltsville, Maryland, U.S.A. (Kwon-Chung, 1975, 2011). Unfortunately, in later papers NIH433, and not NIH430, was indicated to represent the type culture of *F. neoformans* (Kwon-Chung, 1976). According to Martinez et al. (2001) NIH433 is also a serotype D isolate. Kwon-Chung et al. (2011) reported that in the 1975 paper in which *F. neoformans* was described (Kwon-Chung, 1975) compatible crossing were observed between mating-types α and **a** strains of serotype D, as well as between serotype A and serotype D strains, but we could not confirm this when checking the original 1975 publication.

Filobasidiella neoformans variety *neoformans* Kwon-Chung, 1982. In: Kwon-Chung, K.J., Bennett, J.E. & Rhodes, J.C. 1982, Taxonomic studies on *Filobasidiella* species and their anamorphs. Antonie van Leeuwenhoek 48: 25–38. This variety is based on the same holotype as listed above.

Description based on strains CBS882, CBS918, CBS5728, CBS6900, CBS10079 (=WM629), BD5.

After 3 days at 25 °C in 3% glucose medium, sediment is present; yeast cells are globose, subglobose, ellipsoid, to ovoid, 4.0–7.0 × 4.0–6.5 μm, usually with a single bud, but occasionally some cells may adhere. Occasionally yeast cells germinate with a short cylindrical germ tube, 12–15 × 1.2–2.5 μm (i.e., CBS5728). On YMoA, streak colonies are 5.0–6.0 mm width, smooth, moist to mucoid, shiny, creamish white, with an entire margin; cells are broadly ellipsoid, subglobose to globose, 3.5–7.0 μm in diameter, On Dalmau plates only yeast cells present, but CBS5728 showed short filaments. On MEA colonies measure approximately 8 mm diameter, pale yellowish beige (pale isabella), highly mucoid. The *Filobasidiella neoformans* teleomorph has dikaryotic hyphae, clamp connections, cylindrical clavate basidia with four chains with ovoid, ellipsoid, to globose basidiospores that are finely rough (Fraser et al., 2003; Kwon-Chung, 1975; Nielsen et al., 2007; Velagapudi et al., 2009). Serotype D (Belay et al., 1996; Boekhout et al., 1997, 2001; Dromer et al., 1993), genotype AFLP2 (Boekhout et al., 2001), PCR fingerprint and *URA5*-RFLP molecular type VNIV (Meyer et al., 2009, 2011). Clade I in Fig. 1.

5.2.2. Occurrence and habitats

Based on extensive genotyping studies comprising 2755 isolates this species belonged to 5% of the isolates [molecular type VNIV] (Meyer et al., 2011). Among the clinically and veterinary isolates (n = 1250) this was also 5% and among the environmental isolates 4% (Meyer et al., 2011). Only 1.6% of the isolates came from HIV-infected patients and 0.2% from apparently immunocompetent people (Meyer et al., 2011). In some European countries however, the incidence of this species is higher, e.g., the Netherlands 12% (Hagen et al., 2012a), Spain 20% (Guinea et al., 2010), Croatia 40% (Mlinaric-Missoni et al., 2011), Serbia 29% (Arsic Arsenijevic et al., 2014) and Italy 35% (Viviani et al., 2006). *C. deneoformans* is reported to have a global occurrence with Europe as a more prevalent region (Mitchell et al., 2011) and is known from Europe (e.g., Austria, Belgium, Bulgaria, Czech Republic; Denmark, France, Germany, Italy, Netherlands, Portugal, Spain, Switzerland, UK, Russia); Asia

(e.g., China, Thailand); Oceania (e.g., Australia); North America (Canada, U.S.A.); Central and South America (e.g., Brazil, Chile, Colombia, Mexico, Peru) (Escandón et al., 2006; Guinea et al., 2010; Hagen et al., 2012a; Medeiros Ribeiro et al., 2006; Meyer et al., 2011; Viviani et al., 2006). The percentage of this species was reported to range from 4% (Oceania, South America) to 19% (Europe) among environmentally obtained isolates (Meyer et al., 2011, but see above). The higher incidence in some parts of the world, e.g., Europe, was explained by the higher temperature sensitivity of this species if compared to *C. gattii* (Martinez et al., 2001).

The species is known from soil, pigeons and other birds, droppings from pigeon (*Columba livia*), greyish eagle-owl (*Bubo africanus cinerascens*), cuckoo and canary, as well as from cats and dogs (Mitchell et al., 2011). *C. deneoformans* was isolated from a nasal tumor of horse, a dead white mouse, milk from a mastitic cow, and a mastitic cow (Boekhout et al., 2001). The species is also reported from trees, but to a lesser extent than *C. neoformans*. Tree species from which *C. deneoformans* has been isolated belonged to the following families: Chrysobalanaceae (*Licania* (*Moquilea*) *tomentosa*), Fagaceae (*Quercus alba*), and Myrtaceae (*Eucalyptus camaldulensis*, *E. tereticornis*, *Syzygium cucumi*) (Boekhout et al., 2001; Chowdhary et al., 2012b; Colom et al., 2012; Medeiros Ribeiro et al., 2006; Mitchell et al., 2011).

5.2.3. Epidemiology

Infections with *C. deneoformans* seems to be predominant among patients that are immunocompromised, but the species is also causing disease in apparently healthy individuals (Arsic Arsenijevic et al., 2014; Hagen et al., 2012a; Guinea et al., 2010; Mlinaric-Missoni et al., 2011; Sanchini et al., 2014). A correlation between clinical background (patients are more often >60 years of age, skin infections) has been described (Dromer et al., 1996). Higher proportions of *C. deneoformans* infections have been reported among patient cohorts from Europe (Cogliati, 2013; Guinea et al., 2010; Sanchini et al., 2014; Viviani et al., 2006), and smaller percentages from Asia (0.3%), Australia (1%), North America (5%) and South America (1%) as reported in a large global population review (Cogliati, 2013). Epidemiological studies reported that the majority of the *C. deneoformans* isolates had the mating-type α (Arsic Arsenijevic et al., 2014; Bii et al., 2007; Casali et al., 2003; Hagen et al., 2012a; Litvintseva et al., 2005; Sanchini et al., 2014; Viviani et al., 2006; Yan et al., 2002). Although mating-type **a** *C. deneoformans* isolates are less commonly isolated, they are more common than mating-type **a** *C. neoformans* isolates, which are regarded as extremely rare (Arsic Arsenijevic et al., 2014; Feng et al., 2008b; Hagen et al., 2012a; Viviani et al., 2006) (Supplementary Table 4).

5.2.4. Susceptibility to antifungals

Isolates are usually susceptible for the clinically used antifungals amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole, voriconazole and the novel compound isavuconazole (Arsic Arsenijevic et al., 2014; Guinea et al., 2010; Hagen et al., 2012a; Mlinaric-Missoni et al., 2011; Thompson III et al., 2009). Elevated MICs are reported for fluconazole (Hagen et al., 2012a), and a 5-fluorocytosine resistant isolate has been described (Kantarcioglu and Yücel, 2002). Epidemiological cutoff values (ECVs) were recently established, with ECVs for amphotericin being B 1 μg/ml for non-typed *C. deneoformans* isolates; 5-fluorocytosine 16 μg/ml for non-typed *C. deneoformans* isolates; fluconazole 16 μg/ml for non-typed *C. deneoformans* isolates; itraconazole 0.5 μg/ml for non-typed *C. deneoformans* isolates; posaconazole 0.25 μg/ml for non-typed *C. deneoformans* isolates; voriconazole 0.12 μg/ml for *C. deneoformans* isolates; for isavuconazole no *C. deneoformans* specific ECV could be established and therefore the general ECV of 0.06 μg/ml is applied for this species (Espinel-Ingroff et al., 2012a,b, 2015).

5.2.5. Virulence

Cryptococcus deneoformans is more often involved in soft-tissue infections compared to *C. neoformans* (Sanchini et al., 2014). The former species was found to be more susceptible to heat than the latter and this may explain the higher incidence of *C. deneoformans* in skin infections, as well as clearing of this species by fever (Martinez et al., 2001).

5.3. Cryptococcus gattii (Vanbreuseghem & Takashio) Kwon-Chung and Boekhout, 2002 (nomen conservandum, McNeil et al., 2006)

In: Kwon-Chung, K.J., Boekhout, T., Fell, J.W. & Diaz, M. 2002. Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetideae). Taxon 51: 804–806.

Mycobank: MB372381.

Holotype: The dried holotype is not available anymore from the Mycological Department, Institute of Tropical Medicine, Antwerp, Belgium (Kwon-Chung, 2011).

Neotype: CBS H-21966. Ex-type cultures ATCC32269 = CBS6289 = CBS8273 = IHEM11796 = MUCL30449 = NIH3233 = RV20186.

Isolated by E. Gatti & R. Eeckels, June 1966, from spinal fluid of man, Democratic Republic of Congo.

Genotype AFLP4, serotype B, mating-type **a** (Boekhout et al., 2001; Bovers et al., 2008a; Hagen et al., 2010a, 2012b), PCR fingerprinting and *URA5*-RFLP molecular type VGI (Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009).

5.3.1. Confirmed synonyms

Saccharomyces subcutaneous tumefaciens Curtis, 1896. Authentic strain CBS1622, genotype AFLP4, serotype B (Boekhout et al., 2001). In: Curtis, F. 1896, Contribution à l'étude de la Saccharomycose humaine. Ann. Inst. Pasteur 10: 449–468. Invalid name, trinomium.

Cryptococcus hominis Vuillemin variety *tumefaciens* (Curtis) Benham, 1935. Based on the same strain as *S. subcutaneous tumefaciens*.

Cryptococcus hondurianus Castellani, 1933. Authentic strain CBS883, genotype AFLP4, serotype B (Boekhout et al., 2001). In: Castellani, A., 1933. Blastomycosis: a short general account. Med. Press Circul. 136: 438–443. Although this name has priority over *C. gattii* a proposal to conserve the latter over *C. hondurianus* was accepted (McNeil et al., 2006), and the name *C. hondurianus* is rejected.

Torulopsis hominis Vuillemin variety *honduriana* Castellani, 1933. In: Castellani, A., 1933, Observations on fungi isolated from cases of blastomycosis cutis and blastomycosis pulmonalis in North America and Europe. Remarks on blastomycetin. J. Trop. Med. Hyg. 20: 297–321. Based on same authentic strain as C. *hondurianus*.

Torulopsis neoformans variety *sheppei* Giordani, 1935. Authentic strain CBS919, genotype AFLP4, serotype B (Boekhout et al., 2001). In: Giordano, A., 1935, Rôle du *Torulopsis neoformans* (Sanfelice) Red. En pathologie humaine. Boll. Sez. Ital. Soc. Intern. Microbiol. 7: 119–123. [Invalid name, no latin diagnosis]

Cryptococcus neoformans variety *gattii* Vanbreuseghem and Takashio, 1970. Type RV20186, genotype AFLP4, serotype B (Boekhout et al., 2001). In: Vanbreuseghem, R. & Takashio, M., 1970. An atypical strain of *Cryptococcus neoformans* (Sanfelice) Vuillemin 1894. Part II. *Cryptococcus neoformans* var. *gattii* var. nov. Ann. Soc. Belg. Méd. Trop. 50: 695–702.

Cryptococcus neoformans variety *shanghaiensis* Liao, Shao, Zhang & Li, 1983 [nomen inval. no latin diagnosis]. Type CBS7229, genotype AFLP4, serotype B (Boekhout et al., 2001). In: Liao, W., Shao, J., Wu, S., Zhang, J. & Li, S., 1983. *Cryptococcus neoformans* var. *shanghaiensis* caused meningitis. Chin. Med. J. (English edt.) 96: 287–290.
Filobasidiella bacillispora and *Filobasidiella neoformans* var. *bacillispora* Kwon-Chung, 1982 are listed as teleomorphs of *Cryptococcus gattii* (Vanbreuseghem & Takashio) Kwon-Chung & Boekhout (Kwon-Chung, 2011; Kwon-Chung et al., 2002), but this teleomorph is not based on a crossing between *C. gattii* strains, but between a strain of *C. bacillisporus* (CBS6955) and one of *C. deuterogattii* (CBS6956, see below).

Description based on strains CBS6289, CBS7992 (=RV54130), CBS10078 (=WM179), CBS10510 (=WM276), CBS11231 (=56A).

After 3 days at 25 °C in 3% glucose medium, sediment is present; cells are globose, subglobose, ellipsoid, fusoid to ovoid, 4.0–7.0 × 3.5–6.0 μm, usually with a single bud, but occasionally some cells may adhere. On YMoA, streak colonies are 5.0–6.0 mm width, smooth, moist to mucoid, shiny, creamish white, with an entire margin; cells are broadly ellipsoid, subglobose to globose, 4.0–6.0 μm in diameter. On Dalmau plates only yeast cells present. On MEA colonies measure approximately up to 8 mm, pale yellowish beige (pale isabella), and are highly mucoid. Teleomorph unknown, but most likely *Filobasidiella*-like. Genotype AFLP4 (Boekhout et al., 2001), serotype B (Belay et al., 1996; Boekhout et al., 2001; Dromer et al., 1993; Hagen et al., 2010a, 2012b), PCR fingerprinting molecular type VGI (Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009). Diagnosis: Clade D in Fig. 1.

5.3.2. Occurrence and ecology

Based on extensive genotyping studies of 2755 isolates *C. gattii* (genotype AFLP4/VGI) comprised 9% of the isolates (Meyer et al., 2011) with the same percentage among clinically and veterinary isolates (n = 1250) (Meyer et al., 2011). Approximately 6% of the clinical isolates came from apparent immunocompetent patients and 0.4% from HIV-infected patients (Meyer et al., 2011). *C. gattii* is reported as a globally occurring species with Australia being a more prevalent region (Mitchell et al., 2011). Among the species of the *C. gattii* complex, this species is most common in Europe, Australasia and Asia (Chen et al., 2014). The species is known from Europe (e.g., Austria, Belgium, France, Germany, Greece, Italy, Netherlands, Portugal, Spain, Switzerland, UK); Asia (e.g., China, India, Japan, Malaysia, Papua New Guinea/Southeast Asia, Taiwan, Thailand, Vietnam); Oceania (e.g., Australia, New Zealand); Africa (e.g., Congo, South Africa); North America (Canada, U.S.A.); Central and South America (e.g., Argentina, Brazil, Colombia, Cuba, Ecuador, Honduras, Mexico, Paraguay, Peru, Venezuela) (Baró et al., 1998; Campbell et al., 2005; Cattana et al., 2013; Chowdhary et al., 2012a,b; Colom et al., 2012; Escandón et al., 2006; Illnait-Zaragozí et al., 2011; Lester et al., 2011; Meyer et al., 2011). The percentage among environmentally obtained isolates ranged from 1% (South America), 3% (North America), 27% (Europe) to 70% (Oceania) (Meyer et al., 2011). *C. gattii* is reported from trees belonging to various tree families: Annonaceae (*Polyaltha longifolia*), Combretaceae (*Terminalia catappa*), Cupressaceae (*Cupressus sempervirens*), Fabaceae (*Acacia visca*, *A. nilotica*, *Cassia fistula*, *Ceratonia siliqua*, *Tipuana tipu*), Meliaceae (*Azadirachta indica*), Myrtaceae (*Eucalyptus camaldulensis*, *E. citriodora*, *E. glomulifera*, *E. tereticornis*, *Eucalyptus* spec., *Syzygium cumini*), Oleaceae (*Olea* spec.), Pinaceae (*Cedrus deodara*, *Pinus canariensis*, *P. halepensis*, *Pseudotsuga menziesii*), Sapotaceae (*Manilkara hexandra*, *Mimusops elengi*) and Ulmaceae (*Ulmus campestris*). The species is reported from bark hollows, detritus, woody debris and soil near the base of trees (Chowdhary et al., 2012a; Mitchell et al., 2011; Refojo et al., 2009; Springer et al., 2014; Vilcins et al., 2002).

The species is also reported from a variety of animals, such as cats, cheetah, dogs, ferrets, goats (lung, brain, gut), a mastitic cow, veterinary samples (Baró et al., 1998; Boekhout et al., 2001; Campbell et al., 2005; Chowdhary et al., 2012b; Colom et al., 2012; Illnait-Zaragozí et al., 2011; Kidd et al., 2004; Lockhart et al., 2013; Mitchell et al., 2011; Morera et al., 2014; Singer et al., 2014). Ferrets may be asymptomatic carriers (Colom et al., 2012). In Australia, probably many more animal species, such as psittacine birds and koala's harbor this species. Probably a large part of the reports by Australian workers, such as R. Malik and M. Krockenberger, may relate to this species, but due to lack of available genotyping studies this could not be confirmed here.

5.3.3. Epidemiology

Although the majority of infections have been observed to cause disease in apparently healthy individuals (Byrnes III et al., 2009; Chau et al., 2010; McCulloh et al., 2011) it has also been isolated from patients with underlying disease (Hagen et al., 2012b). *C. gattii* cases have been reported in high numbers from Australia (39%), while smaller percentages were found in Africa (1%), Asia (13.2%), Europe (3.4%), North America (7%) and South America (4%) (Cogliati, 2013). Large-scale epidemiological studies reported that the percentage of mating-type **a** isolates can be up to 20–25% of the globally collected *C. gattii* isolates (Fraser et al., 2005; Hagen et al., 2012b). But studies from Australia and the U.S.A. reported less than 7% of mating-type **a** isolates (Campbell et al., 2005; Lockhart et al., 2013) or even mating-type α only as reported from China (Chen et al., 2008; Feng et al., 2008b).

5.3.4. Susceptibility to antifungals

In general isolates of *C. gattii* are susceptible to amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole, voriconazole and the novel compound isavuconazole, when determined according to the CLSI guidelines (Chowdhary et al., 2011; Lockhart et al., 2012). *C. gattii* isolates were found to have the lowest geometric mean MICs for fluconazole compared to *C. deuterogattii*, which had the highest MIC values (Lockhart et al., 2012; Silva et al., 2012; Trilles et al., 2012). This observation was also seen for 5-fluorocytosine, albaconazole and voriconazole (Trilles et al., 2012). When compared to the formerly known *C. neoformans* species complex comprising *C. neoformans* and *C. deneoformans*, the MICs for 5-fluorocytosine and amphotericin B were significantly lower for *C. gattii*. Epidemiological cutoff values (ECVs) have been established for fluconazole (8 μg/ml), itraconazole (0.5 μg/ml), posaconazole (0.5 μg/ml) and voriconazole (0.12 μg/ml) (Lockhart et al., 2012). Another, more extensive, study also assessed ECVs as follows: amphotericin B 0.5 μg/ml, 5-fluorocytosine 4 μg/ml, fluconazole 8 μg/ml, itraconazole, posaconazole and voriconazole 0.5 μg/ml, and isavuconazole 0.25 μg/ml (Espinel-Ingroff et al., 2012a,b, 2015).

5.3.5. Virulence

By using a *Galleria mellonella* virulence model the virulence of *C. gattii*, *C. bacillisporus*, *C. deuterogattii* and *C. tetragattii* was found to be similar for all species, and all these species contained isolates with more virulent phenotypes, as well as avirulent phenotypes (Firacative et al., 2014). Virulence of *C. gattii* (cited as molecular type VGI, Thompson III et al., 2014) as assessed in a *Drosophila* host was lower if compared to *C. bacillisporus* (=molecular type VGIII). Similarly, growth rates at 30 °C were also lower when compared to those of *C. bacillisporus*. Melanisation at 30 °C using L-DOPA medium was found less than that of *C. neoformans* (cited as molecular type VNI, Thompson III et al., 2014). Capsule size was found to be higher than those of *C. deuterogattii* and *C. neoformans*

(Thompson III et al., 2014). Australian cats showed less extensive infection when infected with *C. gattii* if compared to *C. deuterogattii* (O'Brien et al., 2004).

5.4. Cryptococcus bacillisporus Kwon-Chung & Bennett

In: Kwon-Chung, K.J., Bennett, J.E. & Theodore, T.S., 1978, *Cryptococcus bacillisporus* sp. nov.: Serotype B-C of *Cryptococcus neoformans*. Int. J. Syst. Bact. 28: 616–620.

Mycobank: MB312336.

Holotype: ATCC32608. Ex-type material also preserved as CBS H-21967. Ex-type cultures ATCC32608 = CBS6955 = NIH191.

Isolated from spinal fluid of patient with cryptococcal meningitis, California, U.S.A.

Genotype AFLP5, serotype C, mating-type **a** (Boekhout et al., 1997, 2001).

5.4.1. Confirmed synonyms

Filobasidiella bacillispora Kwon-Chung *pro parte*, 1976. Type BPI71855, National Fungus Collection, Beltsville, Maryland, U.S.A., based on cross between NIH191 (=CBS6955 = ATCC32608, genotype AFLP5, serotype C (Boekhout et al., 1997, 2001)) and NIH444 (=ATCC32609 = CBS6956, genotype AFLP6, serotype B (Boekhout et al., 1997, 2001; Kwon-Chung et al., 1978). Thus this teleomorphic species is based on a crossing between *C. deuterogattii* (CBS6956) and *C. bacillisporus* (CBS6955). Therefore, we consider that the offspring of this crossing represented a hybrid progeny.

Note: Cryptococcus bacillisporus Kwon-Chung & Bennett was considered previously a synonym under *C. gattii* (Vanbreuseghem & Takashio) Kwon-Chung & Boekhout with the latter name being conserved and thus having priority (McNeil et al., 2006). However, our current data show that they represent two species, and, therefore, the name *C. bacillisporus* is reintroduced.

Description based on strains CBS8755, CBS6955, CBS10081 (=WM161 = WM175), WM728.

After 3 days at 25 °C in 3% glucose medium a sediment is present; cells are globose, subglobose, ellipsoid, to ovoid, usually with a single bud, but occasionally some cells adhere, 4.0–7.0 × 3.7–6.0 μm; bigger cells measuring 8–12 × 6.0–7.0 μm occur. On YMoA, streak colonies are 4.0–5.0 mm width, smooth, moist to somewhat mucoid, shiny, creamish white, with an entire margin; cells are broadly ellipsoid, subglobose to globose, 3.5–7.5 μm in diameter. Dalmau plates show only yeast cells. On MEA colonies measure approximately up to 8 mm, pale yellowish beige (pale isabella), highly mucoid; cells more ellipsoid to ovoid, 5.0–9.0 × 3.5–7.0 μm, with polar and sympodial budding. Teleomorph with *Filobasidiella*-state with ellipsoid smooth basidiospores (Byrnes III et al., 2011; Fraser et al., 2003; Springer et al., 2014; Velagapudi et al., 2009). Serotype C (Dromer et al., 1993; Boekhout et al., 1997, 2001), genotype AFLP5 (Boekhout et al., 2001; Hagen et al., 2010a, 2012b), PCR fingerprinting and *URA5*-RFLP molecular type VGIII (Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009); MLST VGIIIa and VGIIIb (Springer et al., 2014). Clade C in Fig. 1.

5.4.2. Occurrence and ecology

Cryptococcus bacillisporus comprised 4% of 2755 globally collected cryptococcal isolates (Meyer et al., 2011). Among clinically and veterinary isolates (n = 1250) this was 3% and among environmental isolates 6% (Meyer et al., 2011). Approximately, 2% from 1250 clinical isolates came from apparent immunocompetent patients, and only 0.4% from HIV-infected individuals (Meyer et al., 2011). Among the species of the *C. gattii* species complex this species comprised 11% (Chen et al., 2014). The species is known from Europe (Belgium); Asia (India, Papua New Guinea/Southeast Asia, South Korea), Oceania (Australia, New Zealand), Africa (Botswana, South Africa, Tanzania, Malawi), North America (Canada, and California, U.S.A.); South and Central America (Argentina, Brazil, Colombia, Guatemala, Mexico, Venezuela) (Campbell et al., 2005; Chaturvedi et al., 2005; Chen et al., 2014; Escandón et al., 2006, 2010; Lester et al., 2011; Litvintseva et al., 2005, 2011; Meyer et al., 2011; Springer et al., 2014). The species is reported from some tree families, either directly or from soil near the base of the trees: Altingiaceae (*Liquidambar excelsa*, *L. styraciflua*), Combretaceae (*Terminalia catappa*); Myrtaceae (*Eucalyptus* spec., *Corymbia ficifolia*, *Metrosideros excelsa*) and Pinaceae (*Pinus canariensis*) (Boekhout et al., 2001; Chowdhary et al., 2013; Escandón et al., 2010; Mitchell et al., 2011; Springer et al., 2014). *C. bacillisporus* has also been isolated from cats and a cow (Lockhart et al., 2013; Singer et al., 2014).

5.4.3. Epidemiology

Cryptococcus bacillisporus is frequently isolated from immunocompromised individuals, especially among HIV-infected patients (Byrnes III et al., 2011; Chaturvedi et al., 2005; Souza et al., 2010). Little is known about the global distribution (Chowdhary et al., 2013), but a large-scale epidemiological literature review reported only small numbers from the various continents ranging from only 0.1% from Asia and Europe up to 3% for Australia and 4% for North and South America (Cogliati, 2013). The species has been noted from HIV-infected individuals in Southern California (Byrnes III et al., 2011; Chaturvedi et al., 2005; Springer et al., 2014), where trees have been identified as environmental reservoir. *C. bacillisporus* has also been isolated from HIV-infected individuals in Malawi and Botswana (Litvintseva et al., 2005). Studies that made use of MLST showed the existence of two major clades, named genotypes VGIIIa and VGIIIb (Byrnes et al., 2011; Springer et al., 2014). The study by Springer et al. (2014) revealed the existence of a distantly related isolate that was regarded as being subgenotype VGIIIc, but subsequent re-analysis of the data showed that the isolate clustered together with the reference strain for *C. decagattii* (Springer et al., 2014; this study; Fig. 5, Supplementary Data). When MLST data of *C. bacillisporus* isolates from other recent studies was re-analyzed it appeared that a third subgenotype exists (this study; Fig. 5, Supplementary Data). Two studies that included globally collected *C. bacillisporus* isolates reported 13% to less than 5% of mating-type **a** (Fraser et al., 2005; Hagen et al., 2012b). Lockhart et al. (2013), Byrnes et al. (2011) and Escandón et al. (2006) studied *C. bacillisporus* isolates from the U.S.A. and Colombia and reported mating-type **a** to α ratios of approximately 1–9, 1–5 and 0–1, respectively.

5.4.4. Susceptibility to antifungals

Isolates of *C. bacillisporus* are usually susceptible to amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole, voriconazole and the novel compound isavuconazole, when determined according to the CLSI guidelines (Chowdhary et al., 2013; Lockhart et al., 2012; Souza et al., 2010). MIC values for fluconazole, itraconazole and voriconazole were found to be lower compared to *C. tetragattii* and *C. deuterogattii*), but higher than those of *C. gattii* isolates (cited as molecular types VGIV, VGII, and VGI, respectively, Lockhart et al., 2012). Another study found that the MICs for 5-fluorocytosine and itraconazole were lower than those of *C. neoformans* (Singer et al., 2014). Epidemiological cutoff values (ECVs) have been established for fluconazole (8 μg/ml), itraconazole (1 μg/ml), posaconazole (0.12 μg/ml) and voriconazole (0.12 μg/ml) (Lockhart et al., 2012). ECVs assessed in another, more extensive, study were amphotericin B 1 μg/ml for non-typed

isolates; 5-fluorocytosine 4 μg/ml for non-typed isolates; fluconazole 8 μg/ml; itraconazole 0.5 μg/ml; posaconazole 0.5 μg/ml for non-typed isolates; voriconazole and isavuconazole 0.25 μg/ml for non-typed isolates (Espinel-Ingroff et al., 2012a,b, 2015).

5.4.5. Virulence

Among the *C. gattii* species complex, isolates of *C. bacillisporus* grew the best at 30 °C (Thompson III et al., 2014). Using a *Galleria mellonella* virulence model no significant differences in virulence were found among the four species of the *C. gattii* species complex (Firacative et al., 2014). Virulence of *C. bacillisporus* (cited as molecular type VGIII, Thompson III et al., 2014) as assessed in a *Drosophila* host was highest if compared to *C. neoformans* (=molecular type VNI), *C. gattii* (=molecular type VGI), *C. deuterogattii* (=molecular type VGII) and *C. tetragattii* (=molecular type VGIV *pro parte*). Similarly, isolates of this species showed the highest growth rate at 30 °C. Melanisation at 30 °C using L-DOPA medium was found less than that of *C. neoformans* (cited as molecular type VNI, Thompson III et al., 2014). Capsule size was found to be higher than those of *C. deuterogattii* and *C. neoformans* (Thompson III et al., 2014). Virulence in mice ranged from avirulent to moderately virulent and correlated well with moderate intracellular proliferation rates in macrophages (Springer et al., 2014).

5.5. Cryptococcus deuterogattii Hagen & Boekhout, sp. nov.

Etymology: 'Deutero', Greek for second, referring to the molecular type VGII under which this species is hitherto known; 'gattii' referring to the *Cryptococcus gattii* species complex to which this species belonged to previously.

Mycobank: MB810282.

Holotype: CBS H-21968. Ex-type cultures CBS10514 = CDC-R265 = A1M-R265 = WM02.32 = AMFC2607.

Isolated from bronchial wash of man, Duncan, Vancouver Island, British Colombia, Canada.

Genotype AFLP6, serotype B, mating-type α (Bovers et al., 2008a; Hagen et al., 2010a, 2012b; Kidd et al., 2004).

5.5.1. Confirmed synonym

Filobasidiella bacillispora pro parte (see above under *C. bacillisporus*).

Description based on strains CBS10090 (=AV55), CBS10082 (=WM178), CBS10514 (=CDC-R265 = A1M-R265), CBS10865 (=CDC-R272 = A1M-R272).

After 3 days at 25 °C in 3% glucose medium, a sediment is present; cells are globose, subglobose, ellipsoid, to ovoid, usually with a single bud, but occasionally some cells adhere, 4.0–7.5(−8.5) × 3.8–6.8(−7.8) μm. Bigger cells measuring 9.0–13.0 × 9.0–11.0 μm occur. On YMoA, streak colonies are 5.0–6.0 mm width, smooth, moist to mucoid, shiny, creamish white, with an entire margin; cells are broadly ellipsoid, subglobose to globose, 4.0–8.0 μm in diameter. Dalmau plates show only yeast cells, with those at the outermost margin somewhat smaller. On MEA colonies measure approximately up to 8 mm, pale yellowish beige (pale isabella), highly mucoid. Teleomorph with *Filobasidiella*-state (Kidd et al., 2004; Voelz et al., 2013). Mating experiments using two highly fertile tester strains of *C. bacillisporus* (NIH312, B-4546) revealed a *Filobasidiella* teleomorph with four chains of ellipsoid basidiospores. True clamp connections seem to be present (Ngamskulrungroj et al., 2008). This teleomorph is, however, considered by the present authors to represent the result of an interspecies crossing. Serotype B, genotype AFLP6 (Boekhout et al., 2001; Hagen et al., 2010a, 2012b; Kidd et al., 2004), PCR fingerprinting and *URA5*-RFLP molecular type VGII (Kidd et al., 2004; Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009). Clade A in Fig. 1.

5.5.2. Occurrence and ecology

Cryptococcus deuterogattii is a major emerging pathogen that infects primarily apparent immunocompetent humans and animals and that is causing some major outbreaks, most notably those on Vancouver Island and the neighboring mainland of British Colombia, Canada and the Pacific Northwest, U.S.A. (Kidd et al., 2004; Byrnes III et al., 2011; Fraser et al., 2005; Hagen et al., 2013). The species seems emerging in Mediterranean Europe (Hagen et al., 2012b) and has been found to infect tourists that visited endemic regions (Georgi et al., 2009; Hagen et al., 2010b; Lindberg et al., 2007). *C. deuterogattii* comprised 13% of 2755 globally collected isolates of the *C. gattii*/*C. neoformans* species complex (Meyer et al., 2011). Among the clinically and veterinary isolates (n = 1250) this was 7% but among the 604 environmental isolates 35% (Meyer et al., 2011). Eighteen percent of 1250 isolates came from apparent immunocompetent patients (Meyer et al., 2011). Almost half of the isolates (47%) of isolates of representatives of the *C. gattii* species complex comprised this species (Chen et al., 2014). The species *C. deuterogattii* is known from Europe (e.g., France, Germany, Greece, Netherlands, Spain); Asia (e.g., China, India, Japan, Malaysia, Papua New Guinea/Southeast Asia, South Korea, Thailand, Vietnam); Oceania (e.g., Australia (Northern Territory, Southwest), New Zealand); Africa (e.g., Congo, Senegal, South Africa); North America (Canada, U.S.A.); Central and South America (e.g., Aruba, Argentina, Brazil, Colombia, Costa Rica, French Guiana, Mexico, Puerto Rico, Uruguay, Venezuela) (Campbell et al., 2005; Chen et al., 2014; Datta et al., 2009; Escandón et al., 2006; Hagen et al., 2012b, 2013; Lester et al., 2011; Meyer et al., 2011). The percentage among environmentally obtained isolates ranged from 10% (Oceania), 36% (South America) to 61% (North America) (Meyer et al., 2011). As indicated by these latter authors, the high incidence reported for North America may be related to the ongoing outbreaks that stimulated environmental sampling. The species has also been reported in a number of patients from restricted geographic regions, such as Greece, Rio de Janeiro and Para districts in Brazil, and Japan (Hagen et al., 2012b; Okamoto et al., 2010; Pinto Junior et al., 2010). The species has been reported from a number of plant families: Aceraceae (*Acer* spec.), Betulaceae (*Alnus rubra*, *Alnus* spec.), Cupressaceae (*Cupressus lusitanica*, *Thuja plicata*), Ericaceae (*Arbutus menziesii*), Fabacaea (*Acacia decurrens*, *Cassia grande*, *Senna siamea*), Fagaceae (*Quercus garryana*), Euphorbiaceae (*Croton bogotanus*, *C. funckianus*), Lauraceae (*Laurus* spec.), Leguminosae (*Caesalpinia coriaria*), Myrtaceae (*Eucalyptus camaldulensis*, *E. tetradonta*), Moraceae (*Ficus microcarpa*, *F. soatensis*), Pinaceae (*Cedrus* spec., *Picea* spec., *Pinus radiata*, *Pinus* spec., *Pseudostuga menziesii*), Rosaceae (*Licania* (*Moquilea*) *tomentosa*, *Prunus emargata*, *P. dulcis*) and Rubiaceae (*Guattarda acreanus*). The species is isolated from air, and from bark and hollows, as well as flowers of *Eucalyptus camaldulensis* (Boekhout et al., 2001; Chowdhary et al., 2012b; Fortes et al., 2001; Hagen et al., 2013; Kidd et al., 2004; Mitchell et al., 2011).

Also many animals have been found to be colonized or infected by *C. deuterogattii*, such as alpaca and other camelids, cat, dog, Dall's porpoises, elk, ferret, goat, horse, llama, parrot, sheep, koalas, Spinner dolphin (Hawaii) and veterinary samples. Veterinary sources included tracheal wash, liver, and sinuses. The yeast was also found in the nest of a communal wasp, *Polybia occidentialis*, in Uruguay (Boekhout et al., 2001; Campbell et al., 2005; Cardoso et al., 2013; Duncan et al., 2011; Hagen et al., 2013, 2014; Kidd et al., 2004; Kluger et al., 2006; Lockhart et al., 2013; MacDougall et al., 2007; McGill et al., 2009; Mitchell et al., 2011; Morera et al., 2014; Singer et al., 2014; Springer et al., 2014).

5.5.3. Epidemiology

Cryptococcus deuterogattii is predominantly found in HIV-negative individuals (Chan et al., 2014; Favalessa et al., 2014b; Hagen et al., 2012b; Kidd et al., 2004; Smith et al., 2014; Soares et al., 2008) as well as in apparently immunocompetent animals (Cardoso et al., 2013; Duncan et al., 2005; Lester et al., 2004). However, numerous cases of HIV-positive patients with a *C. deuterogattii* infection have been described as well (Pinto Junior et al., 2010; Poonwan et al., 1997). The species can cause disease among children, especially in the South American Amazonean regions (Debourgogne et al., 2012; Pinto Junior et al., 2010; Santos et al., 2008; Soares et al., 2008). *C. deuterogattii* is frequently reported from Australia (22%), North America (44.2%), South America (17%), and to a lesser extent from Africa (0.25%), Asia (1.7%) and Europe (0.3%), as reported in a large literature review (Cogliati, 2013). A large proportion of the infections reported relate to ongoing outbreaks in North America that have affected the health of hundreds of humans and many more animals (Hagen et al., 2013; Kidd et al., 2004), as well as from other smaller outbreaks (Hagen et al., 2013; Raso et al., 2004). A large proportion of the known mating-type **a** *C. deuterogattii* isolates was reported from the South American continent, here the mating-type α isolates were nearly outnumbered as over 95% of 119 investigated Colombian *C. deuterogattii* isolates were found to be mating-type **a** (Escandón et al., 2006). Another study reported a nearly equal distribution of South American mating-type **a** and α isolates (Hagen et al., 2013). Several studies that included numerous *C. deuterogattii* isolates from the outbreaks at the North American continent have reported that all these isolates were of the α mating-type (Fraser et al., 2005; Hagen et al., 2013; Kidd et al., 2004; Lockhart et al., 2013). Studies that investigated Australian, European and North American reported that the majority of the isolates were mating-type α, ranging from 0% to 12% of mating-type **a** isolates (Byrnes et al., 2010; Campbell et al., 2005; Carriconde et al., 2011; Hagen et al., 2012b).

5.5.4. Susceptibility to antifungals

Isolates of *C. deuterogattii* are usually susceptible to the clinically relevant antifungal compounds amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole and voriconazole (Cardoso et al., 2013; Datta et al., 2013; Favalessa et al., 2014a,b; Lockhart et al., 2012; Poonwan et al., 1997; Tsujisaki et al., 2013). MICs are significantly higher when compared to other species of the *C. gattii* species complex (Datta et al., 2013; Iqbal et al., 2010; Lockhart et al., 2012; Silva et al., 2012), but the MIC for isavuconazole is low (Datta et al., 2013). The geometric mean of the MIC for fluconazole is higher than that of *C. gattii* (Chong et al., 2010; Iqbal et al., 2010; Lockhart et al., 2012; Silva et al., 2012). This was also true for 5-fluorocytosine (Chong et al., 2010; Trilles et al., 2012), and albaconazole and voriconazole (Trilles et al., 2012). Interestingly, significant differences exist between the three subgenotypes of *C. deuterogattii* (Iqbal et al., 2010). Isolates of *C. deuterogattii* were found to be less susceptible to the tested antifungal compounds than of the *C. neoformans* species complex (Trilles et al., 2012). Resistance to fluconazole has been reported (Matos et al., 2012). Epidemiological cutoff values (ECVs) have been established for fluconazole (32 μg/ml), itraconazole (1 μg/ml), posaconazole (1 μg/ml) and voriconazole (0.5 μg/ml) (Lockhart et al., 2012). In another, more extensive, study ECVs were assessed as follows: amphotericin B 0.5 μg/ml for genotype AFLP6A/VGIIa and 1 μg/ml for non-typed *C. deuterogattii* isolates; 5-fluorocytosine 16 μg/ml for non-typed *C. deuterogattii* isolates; fluconazole 8 μg/ml for genotype AFLP6A/VGIIa and 32 μg/ml for non-typed *C. deuterogattii* isolates; itraconazole 0.5 μg/ml; posaconazole 0.5 μg/ml; and voriconazole and isavuconazole 0.25 μg/ml for genotype AFLP6A/VGIIa and non-typed *C. deuterogattii* isolates (Espinel-Ingroff et al., 2012a,b, 2015).

5.5.5. Virulence

Cryptococcus deuterogattii seems to have a preference to cause pulmonary disease and may be associated with certain immunecompromising or otherwise predisposing host factors (Chen et al., 2014; Harris et al., 2011; MacDougall et al., 2007, 2011). By using a *Galleria mellonella* model, virulence did not differ significantly between all species of the *C. gattii* species complex. Isolates of all species can be either highly virulent or avirulent (Firacative et al., 2014). Experiments showed that mice with an AIDS-like disease are less susceptible to infection with *C. deuterogattii* infection than to *C. neoformans* (Leongson et al., 2013). Virulence of isolates of *C. deuterogattii* (cited as molecular type VGII) as assessed in a *Drosophila* host was found to be less than that of *C. bacillisporus* as was the growth rate at 30 °C (cited as molecular type VGIII, Thompson III et al., 2014). Melanisation at 30 °C using L-DOPA medium was found less than that of *C. neoformans* (cited as molecular type VNI, Thompson III et al., 2014). Virulence as observed in mice ranged from avirulent (environmental isolates) to highly virulent and generally correlated well with intracellular proliferation values in macrophages. However, occasionally nonconcordant results were seen in mice and macrophage experiments (Hagen et al., 2013; Ma et al., 2009). Australian cats showed more extensive infection when infected with *C. deuterogattii* if compared to *C. gattii* (O'Brien et al., 2004).

5.6. *Cryptococcus tetragattii* Hagen & Boekhout, sp. nov.

Etymology: 'Tetra', Greek for four, referring to the molecular type VGIV under which this species was known previously; 'gattii' referring to *C. gattii* to which this species belonged previously.

Mycobank: MB810283.

Holotype: CBS H-21969. Ex-type cultures CBS10101 = WM779.

Isolated by V. Davis, 1994, from cheetah, South Africa.

Genotype AFLP7, serotype C, mating-type α (Bovers et al., 2008a; Hagen et al., 2010a, 2012b).

Description based on strains CBS10101 (=WM779), B5742, B5748, M27055.

After 3 days at 25 °C in 3% glucose medium, a sediment is present; cells are subglobose to globose, usually with a single bud, but occasionally some cells may adhere, 4.0–7.5 × 4.0–7.0 μm. On YMoA, streak colonies are 5.0–7.0 mm width, smooth, moist to mucoid, shiny, creamish white, with an entire margin; cells are broadly ellipsoid, subglobose to globose, 4.0–5.5 μm in diameter. Dalmau plates show only yeast cells. On MEA colonies measure approximately 9.0–11.0 mm width, pale yellowish beige (pale isabella), highly mucoid. Teleomorph unknown, but most likely *Filobasidiella*. Serotype C, genotype AFLP7 (Bovers et al., 2008a, 2009; Hagen et al., 2010a, 2012b), PCR-fingerprinting and *URA5*-RFLP molecular type VGIV, although not distinguishable from *C. decagattii* (=genotype AFLP10) by this method (Hagen et al., 2012b; Linares et al., 2015; Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009). Clade E in Fig. 1.

5.6.1. Occurrence and ecology

The notion that *C. tetragattii* is in part identical to molecular type VGIV (see above) makes it difficult, if not impossible, to translate information on occurrence and clinical incidence as reported for molecular type VGIV to *C. tetragattii* and *C. decagattii*, respectively. It is fair to state, however, that both species are relatively rare. The incidence of molecular type VGIV strains among 2755 isolates of the *C. gattii*/*C. neoformans* species complex was only 1% (Meyer et al., 2011). The majority of isolates of the *C. gattii* species complex in Africa seems to belong to this species (Chen et al.,

2014). The species is known from Africa (South Africa, Tanzania), Asia (India), Europe (Spain, Sweden), Central America (Mexico, Puerto Rico), and South America (Argentina, Colombia, Venezuela) (Cogliati, 2013; Cogliati et al., 2012; Hagen et al., 2010a, 2012b; Lester et al., 2011; Linares et al., 2015; Litvintseva et al., 2005; Loperena-Alvarez et al., 2010; Springer and Chaturvedi, 2010). The species [cited as molecular type VGIV] is reported to originate from trees (Mitchell et al., 2011).

5.6.2. Epidemiology

Cryptococcus tetragattii seems rather rare as indicated by the low number of isolates described so far (Cogliati et al., 2012; Hagen et al., 2010a, 2012b; Linares et al., 2015; Litvintseva et al., 2005; Loperena-Alvarez et al., 2010). Mating-type α *C. tetragattii* isolates outnumber those that were mating-type **a** (Hagen et al., 2012b), but other studies reported only mating-type α isolates from Botswana, Malawi and India (Cogliati et al., 2012; Fraser et al., 2005; Litvintseva et al., 2005).

5.6.3. Susceptibility to antifungals

Isolates of *C. tetragattii* are usually susceptible to the clinically relevant antifungals amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole and voriconazole (Lockhart et al., 2012). Isolates of the species were found to have higher geometric mean MICs for fluconazole, itraconazole and voriconazole when compared to *C. gattii* and *C. bacillisporus*, but lower when compared to *C. deuterogattii* (cited with the respective genotypic designations, Lockhart et al., 2012). Epidemiological cutoff values (ECVs) have been established for fluconazole (16 μg/ml), itraconazole (1 μg/ml), posaconazole (1 μg/ml) and voriconazole (0.25 μg/ml) (Lockhart et al., 2012). Another study also assessed ECVs as follows: amphotericin B 1 μg/ml for non-typed *C. tetragattii* isolates; 5-fluorocytosine 4 μg/ml for non-typed *C. tetragattii* isolates; fluconazole 16 μg/ml; itraconazole 1 μg/ml; posaconazole 0.5 μg/ml for non-typed *C. tetragattii* isolates; and voriconazole and isavuconazole 0.25 μg/ml for non-typed *C. tetragattii* isolates (Espinel-Ingroff et al., 2012a,b, 2015).

5.6.4. Virulence

By using a *Galleria mellonella* virulence model it was shown that virulence of *C. gattii*, *C. bacillisporus*, *C. deuterogattii* and *C. tetragattii* did not differ significantly, and in all species highly virulent and avirulent phenotypes are known (Firacative et al., 2014). Virulence of molecular type VGIV strains that represent *C. tetragattii*, namely CBS10101, B5742 and B5748, as assessed in a *Drosophila* host was found to be less than that of *C. bacillisporus* as was the growth rate at 30 °C (cited as molecular type VGIII, Thompson III et al., 2014). Melanisation at 30 °C using L-DOPA medium was found less than that of *C. neoformans* (cited as molecular type VNI, Thompson III et al., 2014).

5.7. Cryptococcus decagattii Hagen & Boekhout, sp. nov.

Etymology: 'Deca' from the Greek word for 10, referring to the AFLP genotype number AFLP10 under which this species was hitherto known; '*gattii*' from *C. gattii*, the species complex where the species belonged to.

Mycobank: MB810284.

Holotype: CBS H-21970. Ex-type cultures CBS11687 = IHEM14941S = IHEM14941 = RV 63979.

Isolated by J. Torres-Rodriguez (Barcelona, Spain) from a HIV-positive man originating from Mexico in 1987.

Genotype AFLP10, serotype B, mating-type **a** (Hagen et al., 2010a, 2012b).

Description based on strains CBS11687 (=IHEM14941S = IHEM14941), IHEM14941W.

After 3 days at 25 °C in 3% glucose medium a sediment is present; cells are broad ellipsoid, ovoid, subglobose to globose, usually with a single bud, but occasionally some cells adhere, 4.0–7.5 × 3.5–6.0 μm. Bigger cells measuring up to 10 × 8 μm occur. On YMoA, streak colonies are 6.0–9.0 mm width, smooth, moist to mucoid, shiny, creamish white, with an entire margin; cells are broad ellipsoid to fusoid, subglobose to globose, 3.5–7.5(−9.0) μm in diameter. Dalmau plates show only yeast cells. On MEA colonies measure 10–15 mm, pale yellowish beige (pale isabella), highly mucoid. Teleomorph unknown, but most likely *Filobasidiella*-like. Serotype B, genotype AFLP10 (Bovers et al., 2008a, 2009; Hagen et al., 2010a, 2012b), PCR fingerprinting molecular type VGIV, although not distinguishable from AFLP7 by this method (Hagen et al., 2012b; Meyer et al., 2009, 2011; Ngamskulrungroj et al., 2009); MLST VGIIIc (Springer et al., 2014); Clade B with CBS11687 in Fig. 1. AFLP genotyping, multi-locus sequence typing (Meyer et al., 2009) and MALDI-TOF MS separate *C. decagattii* from the closely related siblings *C. bacillisporus* (genotype AFLP5/VGIII) and *C. tetragattii* (genotype AFLP7/VGIV) (Hagen et al., 2012a; Linares et al., 2015; Trilles et al., 2014).

Data of isolates with a *URA5*-RFLP determined molecular type VGIV that fell into a putative genotype AFLP5/VGIII cluster based on MLST (marked as VGIII* by Trilles et al., 2014) was re-analyzed together with the two available *C. decagattii* isolates (Hagen et al., 2012b). This revealed that one of these previously published MLST genotype VGIII* isolates was closely related to *C. decagattii* (Fig. 5). Interestingly, this isolate WM1802 (=LA390; mating-type **a**, serotype B) was cultured from a clinical source that had a Mexican origin (Ngamskulrungroj et al., 2009; Trilles et al., 2014). A recent MLST study on molecular type VGIII isolates from Southern California (U.S.A.) showed that one isolate formed a long basal branch (named molecular type VGIIIc by the authors) to a tighter cluster with molecular type VGIIIa and VGIIIb isolates (Springer et al., 2014). The branch length presented in the study by Springer et al. (2014) support, in our opinion, the taxonomic status of the clinical isolates 7685027 and IHEM14941 (=CBS11687) as a distinct species. The MLST data from the atypical molecular type VGIIIc clinical isolate 7685027 (mating-type α) were re-analyzed as described above for the other molecular type VGIII* isolates, and this revealed that this isolate was closely related to the two available *C. decagattii* isolates, as well as to isolate WM1802 (Fig. 5; Supplementary Data). Clade B in Fig. 1.

5.7.1. Occurrence and ecology

Cryptococcus decagattii is a rare species, currently five isolates are known: CBS11687 (=IHEM14941S = IHEM14941) and IHEM14941W (both Hagen et al., 2012b), 7685027 (Springer et al., 2014), WM1802 (=LA390) and WM11.135 (both Trilles et al., 2014). As indicated under *C. tetragattii* this species cannot be distinguished from that species by conventional PCR-fingerprinting and *URA5*-RFLP. Hence it is impossible to directly interpret most data on the distribution and clinical occurrence using strain designations based on PCR fingerprinting.

5.7.2. Epidemiology

Cryptococcus decagattii was originally reported from a HIV-infected individual that originated from Mexico (Hagen et al., 2010a, 2012b). The epidemiology of this species is not clear as the species cannot be separated from *C. tetragattii* when *URA5*-RFLP is applied (Hagen et al., 2010a, 2012b; Trilles et al., 2014). Re-analysis of published MLST-data revealed the existence of two other *C. decagattii* isolates, namely mating-type α isolate 7685027 (Springer et al., 2014) and mating-type **a** WM1802 (=LA390; Trilles et al., 2014) (Fig. 5), the mating-type **a** to α ratio is 3:1 (this study; Hagen et al., 2012b).

5.7.3. Susceptibility to antifungals

Isolates of *C. decagattii* are susceptible to the antifungals amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole, voriconazole and isavuconazole (Hagen et al., 2010a). Epidemiological cutoff values (ECV) could not be determined for *C. decagattii*, and, therefore general ECVs are used as follows: amphotericin B 1 μg/ml; 5-fluorocytosine 4 μg/ml; fluconazole 16 μg/ml; itraconazole 1 μg/ml; posaconazole 0.5 μg/ml; and voriconazole and isavuconazole 0.25 μg/ml (Espinel-Ingroff et al., 2012a,b, 2015).

5.7.4. Virulence

No data have been reported using vertebrate hosts. As indicated above a *Galleria mellonella* virulence model did not show significant differences between the species of the *C. gattii* complex (Firacative et al., 2014).

Conflict of interest

All authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.02.009.

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